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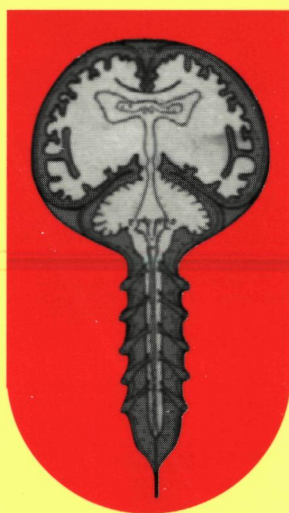
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# Cerebrospinal fluid analysis

Metabolic, brain damage and immunologic aspects



Karel Lamers



## **CEREBROSPINAL FLUID ANALYSIS**

**metabolic, brain damage and immunologic aspects**





# **CEREBROSPINAL FLUID ANALYSIS**

**metabolic, brain damage and immunologic aspects**

**EEN WETENSCHAPPELIJKE PROEVE OP HET GEBIED  
VAN DE MEDISCHE WETENSCHAPPEN**

**PROEFSCHRIFT  
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR  
AAN DE KATHOLIEKE UNIVERSITEIT NIJMEGEN,  
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN  
IN HET OPENBAAR TE VERDEDIGEN OP  
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*Dedicated to my mother;  
in memory of my father,  
my sister Yvonne and my  
brother Wim.*

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**ABBREVIATIONS**

AcAc	acetoacetate
AIDS	acquired immunodeficiency disease
Alb	albumin
ALS	amyotrophic lateral sclerosis
BBB	blood brain barrier
BCB	blood cerebrospinal fluid barrier
$\beta$ -OH-B	$\beta$ -hydroxybutyrate
C3/C4	complement component 3 and 4
CEA	carcino embryonic antigen
CD	cluster determination
CNS	central nervous system
CP	chronic progressive
CSF	cerebrospinal fluid
CVA	cerebrovascular accident
DD	disease duration
DTPA	diethylene triamine-pentaacetic acid
ECF	extracellular fluid
EDSS	Kurtzke's expanded disability status scale
GABA	gamma aminobutyric acid
Gd	gadolinium
GFA	glial fibrillary acidic protein
5-HIAA	5-hydroxyindole acetic acid
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
HVA	homovanillic acid
IEF	isoelectric focusing
Ig	immunoglobulin
IV	intravenous
KJ	kilo joules

<b>L/P</b>	<b>lactate/pyruvate</b>
<b>MAG</b>	<b>myelin associated glycoprotein</b>
<b>MBP</b>	<b>myelin basic protein</b>
<b>MOPEG</b>	<b>3-methoxy-4-hydroxyphenylethylene glycol</b>
<b>MP</b>	<b>methylprednisolone</b>
<b>MP</b>	<b>median percentage</b>
<b>MRI</b>	<b>magnetic resonance imaging</b>
<b>MS</b>	<b>multiple sclerosis</b>
<b>NEFA's</b>	<b>non-esterified fatty acids</b>
<b>NSE</b>	<b>neuron specific enolase</b>
<b>OB</b>	<b>oligoclonal band</b>
<b>PACIA</b>	<b>particle counting immuno assay</b>
<b>PB</b>	<b>peripheral blood</b>
<b>PFS</b>	<b>primary fibromyalgia fibrositis syndrome</b>
<b>PR</b>	<b>progression rate</b>
<b>Q</b>	<b>quotient</b>
<b>RIA</b>	<b>radio immuno assay</b>
<b>RIND</b>	<b>reversible ischemic neurological deficit</b>
<b>RR</b>	<b>relapsing remitting</b>
<b>S-100</b>	<b>S-100 protein</b>
<b>SAM</b>	<b>S-adenosyl methionine</b>
<b>SLE</b>	<b>serum lupus erythematosus</b>
<b>SSPE</b>	<b>subacute sclerosing panencephalitis</b>
<b>T</b>	<b>Tesla</b>
<b>TE</b>	<b>echo time</b>
<b>TIA</b>	<b>transient ischemic attack</b>
<b>TR</b>	<b>repetition time</b>
<b>VMA</b>	<b>vanilmandelic acid</b>





# **CHAPTER 1**

## **GENERAL INTRODUCTION**



---

## GENERAL INTRODUCTION

### 1. Physiology of the cerebrospinal fluid (CSF)

The CSF has 2 anatomical locations: the ventricular one and the subarachnoid one. The extracellular fluid of the brain (ECF), the neurone's environment, is in direct anatomical continuity with the CSF and the exchange between ECF and CSF occurs via patent extracellular channels in the ependyma and appears to be relatively unrestricted. The ease of material exchange between CSF and brain suggests that the fluid in both compartments (ECF and CSF) are similar in composition and that CSF may provide an important route of material access to or exit from brain.

#### *The CSF formation*

The rate of CSF formation in adult man has been established between 0.35 and 0.40 ml/minute (= 600 ml/day). The total CSF volume is about 150 ml. This means that CSF volume is daily replaced 4 times. In (young) children the CSF volume and daily CSF production are lower. In figure 1 the general relations of blood, brain, ECF and CSF are shown by a scheme, where the ventricular fluid is regarded as a single compartment containing a single choroid plexus. The blood comes into relation with the brain in two ways; firstly through the highly vascularized choroid plexuses and secondly through the capillaries of the brain parenchyma. Current evidence suggests that  $\pm 70\%$  of the CSF is produced through secretion by the choroid plexuses and  $\pm 30\%$  from the capillary bed of brain and meninges. The epithelial cells of the choroid plexus are structurally polarized. The basolateral surface is in contact with the blood capillaries, the apical surface with the ventricular CSF via their villi. The rate of the active sodium transport from the epithelial cells of the choroid plexus into the ventricles, due to the presence of a  $\text{Na}^+\text{K}^+\text{ATPase}$  pump, located at the apical surface of these plexus cells, regulates the movement of negatively charged ions and water (osmotic balance) and determines the CSF formation (figure 2).

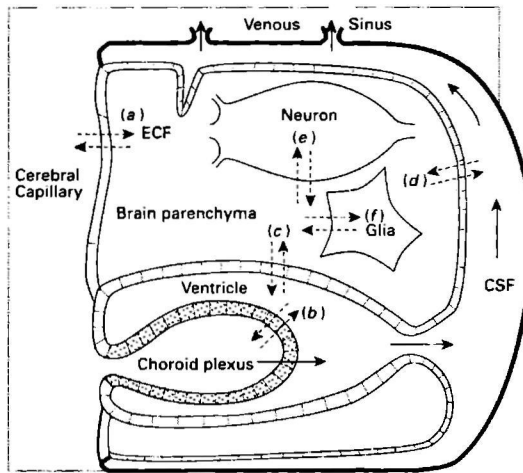


Figure 1: Diagram of fluid compartments of the blood-brain-CSF system. Continuous arrows represent proven directions of CSF flow. Interrupted arrows indicate where diffusion of water and solutes may occur between the different compartments: (a) across the blood-brain barrier, between brain capillaries and extracellular fluid; (b) across the epithelia of the choroid plexuses; (c) across the ependyma; (d) across the pia mater; (e) and (f) across the cell membranes of neurons and glial cells. Thick outline represents the arachnoid-dural enclosure of the system. (With permission from Davson H. In: *Physiology and Pathophysiology of the cerebrospinal fluid*. Editors: Davson H, Welch K, Segal B, Churchill Livingstone, London 1987 p 10).

### *Blood brain/CSF barrier*

Adjacent choroid epithelial cells are sealed together by tight junctions that impede passage of both small molecules and proteins, creating the blood CSF barrier (BCB). Similar junctions exist between the endothelial cells lining the cerebral capillaries, creating the blood brain barrier (BBB). The tight junctions of the epithelial cells are a little more "leaky" than the ones present between endothelial cells of blood capillaries and they restrict the passage of blood proteins into CSF in function of their size and molecular weight. The entry of small molecules and nutrients into the brain is regulated by specific transport systems at both barriers. The BBB and BCB do not function in the same way. The BBB of the cerebral capillaries is principally in charge of transporting those substances which the brain consumes rapidly and in large quantities like: glucose, aminoacids, ketones. The transport is a facilitated diffusion system, which moves molecules directly and quickly into the brain without consuming energy. In contrast, the BCB of the plexus epithelium transfers "micro nutrient"

substances directly into the CSF. Most substances are essential to the brain but in relatively small amounts over extended periods like: vitamins B6 and C, folate and nucleosides. In figure 2, the flow of molecules across the BCB and the regulating transport processes are presented. The transport of these "micro nutrient" substances is mostly an active energy consuming system.

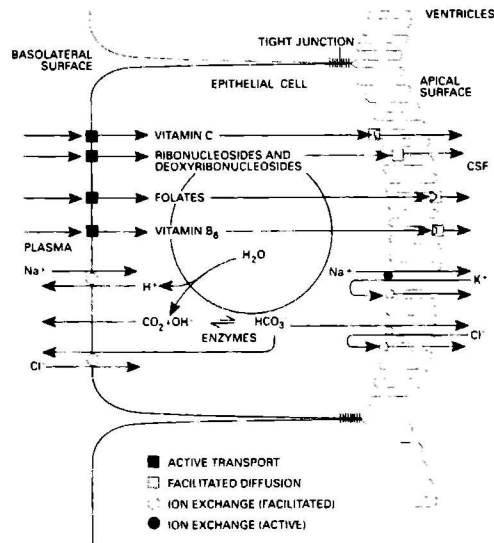


Figure 2. Flow of molecules across the blood-CSF barrier is regulated by several mechanisms in the choroid plexus. Some micronutrients, such as vitamin C, are pulled into the epithelial cells at the basolateral surface by an energy-consuming process known as active transport; the micronutrients are released into the CSF at the apical surface by another regulated process, facilitated diffusion, which requires no energy. Essential ions are also controllably exchanged between the CSF and blood plasma. Transport of an ion in one direction is linked to the transport of a different ion in the opposite direction, as in the exchange of sodium (Na<sup>+</sup>) ions for potassium (K<sup>+</sup>) ions. (With permission from Spector R. In: Scientific American, November 1989).

### *The CSF absorption*

In a steady state, by definition, the rate of absorption of CSF equals its rate of formation. The bulk flow reabsorption of the CSF into the venous system takes place in the pacchionian granulations and arachnoid villi, functioning like a system of one-way valve (figure 1). The choroid plexus has an important role in removing waste products from the CSF into the blood by an active transport system such as: small inorganic ions, drugs or metabolic derivatives of neurotransmitters (figure 2). Finally,

some solutes disappear by diffusion from the CSF into the adjacent brain and capillaries to be removed by the circulation like: glucose and lactate. This last flow reflects the bidirectionality of the carrier mediated transport system of the brain capillaries.

### *The functions of the CSF*

Four major functions of the CSF have been defined:

1. to surround, support and protect nervous system, whereby the delicate neural tissue is cushioned from external forces;
2. to control the microchemical environment of nervous tissue in continuous unrestricted exchange with the ECF;
3. to remove products of cerebral metabolism, serving as a "lymphatic like" drainage system and "sink action" of the brain;
4. to distribute biologically active substances within the central nervous system (CNS) and to provide special intracerebral transport.

### **2. The progress in CSF analysis**

Until 15 years ago CSF analysis for clinical practice was limited to a restricted number of determinations such as: cytology, protein and glucose content, protein electrophoresis, microbiological and serological tests. From the middle of the seventies the interest for measuring more specific CSF components and the importance of CSF/blood protein relations increased. The necessity for simultaneous blood and CSF measurements in order to evaluate an abnormal composition of the CSF became clear. With the introduction of new sensitive micromethods, the used CSF volume for component analysis decreased significantly and therefore the possibility for extension of diagnostic tests increased. The interest for investigating humoral immunological processes in inflammatory neurological diseases such as: multiple sclerosis (MS) or infections with CNS involvement (human immunodeficiency virus (HIV)-infect or neuroborreliosis) by way of CSF analysis of immunoglobulines and specific antibodies was obvious. Also the interest for the investigation of the cellular immune response in the CSF increased by determining subpopulations of immunocompetent cells in CSF

and soluble products (cytokines) of these cells. The importance of the assay of brain specific proteins in CSF as markers of brain damage increased. The appearance of more metastatic malignities in the CNS during the last years determined the importance for tumourmarker investigations in CSF. Furthermore, especially in neurogeriatric patients, studies were performed on abnormalities in vitamins and neurotransmitter metabolites in CSF. Finally, CSF studies in neurometabolic disorders met also increasing interest.

### *The outline of this study*

Books have appeared about (new) CSF analyses. Most books deal with general information about the physiology of the CSF or CSF abnormalities in different neurological diseases. Publications mostly deal with the description of CSF abnormalities in some chemical or cellular components in distinct neurological diseases.

This thesis presents an overview of convential and new CSF components and the diagnostic value of these investigations (chapter 2). In the same chapter a proposal for a strategy of CSF investigation is presented. The different CSF components are subdivided in 9 diagnostic groups. Each group consists of a number of coherent CSF components which define a special aspect of the (abnormal) CNS metabolism. The main point of this thesis concerns studies on the relevance of CSF analyses with respect to 4 diagnostic groups for the neurological diagnostics. The results are presented in the next 3 chapters:

- chapter 3: brain energy metabolism in young neurological patients after fasting;
- chapter 4: brain specific proteins in neurological patients;
- chapter 5: humoral and cellular immunology in neurological patients with an inflammatory disease.

The choice for these 4 diagnostic groups was based on the following considerations:

1. These components might be relevant not only for diagnostics but also for prognostics and for studying medication effects.



2. In various neurological diseases the clinicians need objective parameters for assessing the disease activity.
3. Studies about these components are closely linked with important research lines within our neurological department.

## **CHAPTER 2**

### **CSF DIAGNOSTICS: CLINICAL CHEMICAL AND CLINICAL ASPECTS**

**K.J.B. Lamers and R.A. Wevers**

**TJDSCHRIFT NED. VER. KLIN. CHEM. 1992;17:110-123**

## SUMMARY

Cerebrospinal fluid (CSF) and brain extracellular fluid are in direct contact with each other. Therefore CSF can provide information about the metabolism of the brain. We have developed a strategy for CSF investigation in which various aspects of (abnormal) brain metabolism can be studied. Brain-specific proteins in CSF reveal information about the (degree of) cell damage in the central nervous system (CNS); isoelectric focusing (IEF) and Ig indices about intrathecal humoral immune response; lymphocyte markers on cellular immune response; tumour markers on metastases into CNS; neurotransmitter metabolites and central monoaminergic activity; vitamin deficiencies; metabolic products within the CNS. Abnormalities in the composition of CSF are commonly found in patients with neurological diseases.

We describe the determination of the above-mentioned chemical and cellular components in CSF and their value for differential neurological diagnostics.

---

## DIAGNOSTIC CSF ANALYSIS

### *Introduction*

There is direct contact between the cerebrospinal fluid (CSF) and extracellular fluid of the brain. It is generally accepted that CSF reflects the metabolism of the brain and spinal cord. About 70% of the CSF is formed by the choroid plexus; the rest is formed extrachoroidally by the brain in particular via the brain capillaries, the ependyma of the ventricles and via the blood vessels entering the subarachnoidal space through their walls. About 600 ml of CSF is produced per day. Production and absorption are in equilibrium. Bulk absorption of CSF takes place via the arachnoid villi into the venous system. This absorption is unidirectional and non-selective. On the basis of a total volume of 150 ml, the CSF is renewed about four times per day. A minor part of CSF absorption takes also place via the cervical and lumbar roots into the lymphatic system. In addition, certain products are removed actively by the choroid plexus like: small inorganic ions and drugs and finally products diffuse into the brain and are removed via capillary endothelial cells.

The term 'blood-brain barrier' (BBB) describes the dynamic equilibrium and the exchange of components between the blood, brain and CSF. The term covers the blood-CSF barrier (BCB) and the BBB, but both barriers are not identical. Various systems are responsible for the transport of substances across the BBB. There are carrier-dependent transport systems for the hexose components, neutral amino acids, alkaline amino acids, acid amino acids, monocarboxylic acids, nucleosides and purines. Vitamins and hormones are also transported via carriers. Proteins from the blood diffuse through the tight junctions located between endothelial cells into brain and CSF. Besides the interaction between the CSF and extracellular fluid of the brain, the composition of the CSF components (proteins and low molecular substances) is defined by the concentration of the blood components via the transport functions of the BCB. Correct evaluation of (abnormal) CSF constituents can only be made if blood and CSF are sampled and analysed simultaneously. Biochemical abnormalities in the CSF resulting from abnormal brain metabolism can then be distinguished from abnormalities in the CSF constituents resulting from blood and/or BCB disturbances.

Over the past 10 years, many studies have been published on the value of CSF for the diagnosis of diseases of the central nervous system. The great potential of CSF diagnostics at the present time encouraged us to develop a special strategy to ensure an adequate request policy. For this purpose, we divided the request form and laboratory procedures into 9 diagnostic (sub)groups, so that the physician can select and indicate the aspects of interest for biochemical analysis, in order to support or exclude a special neurological diagnosis:

- Group 1: cells, blood pigments, BCB.
- Group 2: minerals.
- Group 3: brain-specific proteins.
- Group 4: humoral immunology.
- Group 5: cellular immunology.
- Group 6: brain metastases / tumour markers.
- Group 7: neurotransmitters.
- Group 8: vitamins.
- Group 9: metabolic investigations.

These diagnostic (sub)groups are discussed with regard to the type of analysis and the diagnostic value.

#### **Group 1: cells, blood pigments, BCB**

##### **Cells**

Cell counting and cytological examination should be done as quickly as possible after the lumbar puncture. Cell number can be counted in a Fuchs-Rosenthal chamber using phase contrast microscopy. A leukocyte number of  $\leq 4/\mu\text{l}$  is normal. Cytological examination is of diagnostic value also in patients with normal cell number and it is valuable in the differential diagnosis. It is necessary to enrich the cells using, for example, a cytocentrifuge (cytospin), Sayk sedimentation chamber or membrane filtration.

##### **Survival of CSF cells**

Red and white cells are very fragile in CSF after a lumbar puncture. At room

temperature, cell loss, especially granulocytes, can be expected within 2 hours and changes occur in cell morphology. Therefore, CSF for cell investigation must be analysed within 2 hours after puncture, preferable already within 30 minutes.

### Diagnostic value

It is generally accepted that lymphocytes and monocytes in CSF have a haematogenic origin. Pericytes, microglia cells and macrophages in the brain and CSF are transformed blood monocytes. In normal CSF the lymphocyte to monocyte ratio is about 7:3. Small quantities of lymphocytes and monocytes are also present in the connective tissue of the choroid plexus and the leptomeninges in a similar ratio as that found in CSF. Local inflammation causes a sharp increase in the number of cells and more rapid cell transformation. In pathological conditions, white blood cells also appear in the CSF from the brain compartment. The cells disappear from the CSF through degeneration or disperse with the blood via the arachnoid villi and possibly via the lymphatic system.

The following cells can be found in (ab)normal CSF:

#### a. Round (immunocompetent) lymphocytes

- Small lymphocytes. These are seen in viral infection, including human immunodeficiency virus (HIV) infection and in multiple sclerosis (MS). We discuss this subject further under the heading cellular immunology.
- Large stimulated lymphocytes (lymphoid cells). These cells indicate a pathological cellular or humoral reaction.
- Very occasionally, plasma cells are seen in neurological disorders like: MS, neuroborreliosis, neurosyphilis, herpetic encephalitis.

#### b. Mononuclear phagocytes

- Monocytes are often present in normal CSF. There is an increase in the number of cells in the case of infection, ischemia, neoplasia, trauma and haemorrhage.
- Activated monocytes are larger than normal monocytes and display vacuoles

without phagocytosed material. Their presence indicates a reaction to an infection or meningeal stimulation (e.g. myelography).

- Macrophages are activated monocytes with phagocytosed material. They can be differentiated on the basis of the phagocytosed material and can include lipophages, erythrophages (12-18 hours after haemorrhage) and siderophages with haemosiderin, i.e. a haemoglobin degradation product. The latter macrophage appears 6 to 8 days after haemorrhage and can still be encountered several weeks afterwards. Leukocytophages may also be present as a reaction to infection or trauma.

#### c. Granulocytes

Normal CSF does not contain granulocytes. Very sporadically, 1 granulocyte/ $\mu$ l may occur. CSF pleocytosis with granulocytes is very suspicious of acute bacterial infection of the brain and/or meninges (the number of cells can increase to 20,000 leukocytes/ $\mu$ l within a few hours) or an early stage viral infection. An increased number of granulocytes can also occur after meningeal stimulation, trauma, infarction and haemorrhage.

#### d. Eosinophils

Normal CSF does not contain eosinophils. A strong eosinophilic reaction is found particularly in association with parasitic diseases, such as cysticercosis and trichinosis (5% to 30%). Slight eosinophilia (2% to 4%) may also occur after viral infection and meningeal stimulation. Slight eosinophilia is therefore of little diagnostic value.

#### e. Tumour cells

Tumour cell examination in CSF requires specialized knowledge of cell morphology and cell pathology. This test can only be carried out by personnel who have wide experience with the examination of pathological cells. A study by Glass [1] on the correlation between the presence of malignant cells in CSF and the pathological substrate at autopsy of 117 brains of patients with brain tumours (mainly metastases) showed that 26% of the cases had malignant cells in the CSF.

## **Blood pigments**

To distinguish intracranial or intraspinal haemorrhage from traumatic puncture, it is necessary to collect CSF in 3 separate tubes. In the case of traumatic puncture, the CSF will become steadily clearer and the number of cells will decrease from the first to the third tube. This is not the case with haemorrhage, where the colour of the fluid and number of cells remain about the same in all 3 tubes. Lysis of erythrocytes occurs within 2 to 4 hours, which releases blood pigments into the CSF. After the CSF cells have been centrifuged, traumatic puncture reveals a clear supernatant and haemorrhage usually a coloured supernatant. Three major pigments may be present after haemorrhage: oxyhaemoglobin, bilirubin and methaemoglobin.

### *Oxyhaemoglobin*

Oxyhaemoglobin is red and, after dilution, pink. Within 2 hours after the onset of haemorrhage in the subarachnoid space, oxyhaemoglobin will be released from erythrocytes to a maximum level during the first 36 hours. After 7 to 10 days the protein has disappeared.

### *Bilirubin*

Bilirubin is yellow. It is an iron-free derivative of haemoglobin which is formed by macrophages and other cells in the leptomeninges by means of degradation of haemoglobin by the enzyme haeme-oxygenase. Bilirubin does not appear until about 10 hours after the onset of haemorrhage with a maximum of 48 hours. It may still be present 2 to 4 weeks later. Bilirubin in the CSF after haemorrhage must be distinguished from an increased CSF bilirubin level resulting from bilirubinemia (icterus and haemolysis in newborns) or a (strongly) increased CSF total protein level ( $> 2000$  mg/l). After the exclusion of the latter causes, the bilirubin level in CSF can reflect the severity of the meningeal symptoms after subarachnoid haemorrhage.

### *Methaemoglobin*

Methaemoglobin is dark brown. It is a product of haemoglobin and is characteristic of an encapsulated subdural haematoma.



### Blood pigment analysis

**Method:** The supernatants of 2 or 3 tubes are mixed for spectroscopic examination. An extinction of  $< 23 \text{ mE}$  at 415 nm excludes the presence of blood pigments. If the extinction value is higher an absorption spectrum is performed between 400 and 600 nm and the levels of haemoglobin and/or bilirubin can be calculated.

### Diagnostic value

If CSF only contains oxyhaemoglobin, this indicates traumatic puncture or intracranial haemorrhage which occurred less than 2 to 4 hours before the lumbar puncture. If oxyhaemoglobin and bilirubin are present, a haemorrhage is the cause in 95% of the cases. The presence of erythrophages in the cell composition provides additional evidence for haemorrhage. The more bilirubin present (compared to oxyhaemoglobin) the 'older' the haemorrhage. Computer tomography is an accurate method for detecting or excluding haemorrhage, but it is well-known that haemorrhagic infarcts can give rise to isodensity [2]. CSF analysis, particularly a combination of spectroscopic examination and cytology can make an important additional contribution to the diagnosis of intracranial haemorrhage.

### **Blood CSF barrier**

Permeability disturbance of the BCB can be determined in two ways: the determination of the total protein content of the CSF or the CSF/serum albumin ratio (Q alb). The former parameter is generally used, but it has several disadvantages: the protein content can also be raised by immunoglobulin production within the CNS, cell degeneration within the brain and an increased blood-protein level. An increased albumin ratio only occurs in the case of increased permeability of the BCB. Therefore, the albumin ratio is a better parameter than the total protein content. The permeability of the BCB for blood proteins depends on the molecular size and the hydrodynamic radius of the protein [3]. In newborns, the BCB is not yet fully developed and therefore albumin ratio of the CSF is high. The BCB is mature at the age of 3 months. Permeability decreases steadily over the next two years, after which it gradually increases with age. Statz [4] published reference values for the total CSF

protein content and albumin ratio in children and Tibbling [5] in adults (see Table 1). Various neuropathological circumstances can cause increased permeability of the BCB, such as anoxia, ischemia, intoxication, inflammatory reactions and head injuries. Slight permeability disturbances ( $Q_{alb}$  to  $10 \times 10^{-3}$ ) occur in e.g. MS, amyotrophic lateral sclerosis (ALS) and chronic HIV encephalitis; moderate BCB disturbances ( $Q_{alb}$  to  $20 \times 10^{-3}$ ) in e.g. viral meningitis, diabetic polyneuropathy, brain infarcts and meningoencephalitis; severe BCB disturbances ( $Q_{alb} > 20 \times 10^{-3}$ ) in e.g. the Guillain Barré syndrome, neuroborreliosis, herpes simplex encephalitis and bacterial, including tuberculous, meningitis.

Table 1. Reference values in CSF: total protein and CSF serum albumin ratio

	CSF total protein (mg/l)		CSF/serum albumin ratio ( $\times 10^{-3}$ )	
	mean	range	mean	range
0- 1 week	770	450-1090	12.6	5.6-23.2
1- 4 weeks	660	510-1010	10.2	7.6-16.4
1- 3 months	450	240- 650	5.3	2.3-10.6
3- 6 months	290	230- 370	3.1	2.0- 4.8
6-12 months	270	170- 350	2.5	1.4- 4.5
1-10 years	220	160- 310	1.9	1.0- 4.5
11-18 years	250	160- 400	2.3	1.0- 5.0
		$\pm 2 Sd$		$\pm 2 Sd$
18-30 years	360	240- 490	3.7	1.7- 5.7
31-40 years	360	240- 490	4.0	1.8- 6.2
41-50 years	430	270- 600	4.6	2.0- 7.2
51-60 years	480	290- 670	5.5	2.1- 8.9
61-70 years	530	262- 790	5.6	2.2- 9.9

## Group 2: minerals

### Sodium

The sodium concentration in CSF (144-152 mmol/l) is slightly higher than in

plasma. The choroidal CSF production is determined to a large extent by the blood sodium transport across the BCB. CSF concentration depends strongly on the blood concentration and has little clinical significance.

### *Potassium*

The potassium concentration in CSF (2.7 to 3.1 mmol/l) is lower than in plasma. The concentration is extremely constant and changes in plasma concentrations have little influence on the CSF concentration. Sodium/potassium ATPase which is involved in the active transport of potassium in the choroid plexus, plays an important role in CSF potassium homeostasis. An active transport system seems present in the brain which transports (excess) potassium e.g. after haemorrhage to the blood.

As potassium has a depolarizing effect on cell membranes, the brain compartment benefits from adequate regulation to maintain a stable potassium concentration.

### *Calcium*

The calcium concentration in CSF (1.02 to 1.18 mmol/l) is considerably lower than in plasma. The concentration is determined by carrier dependent transport. The clinical significance of the CSF calcium concentration is not yet clear.

### *Phosphate*

The concentration in CSF (0.40 to 0.55 mmol/l) is about 50% of the plasma concentration. CSF concentration is independent of the plasma concentration. Increased CSF phosphate levels are only observed if the CSF protein concentration is very high (e.g. BCB disturbance).

### **Group 3: brain-specific proteins**

In brain, there are a number of brain-specific proteins which are characteristic for certain cell types, e.g. S-100 and glial fibrillary acidic protein (GFA) are present in astrocytes, myelin basic protein (MBP), proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) in oligodendroglia and myelin and neuron-specific

enolase (NSE) in neurons. If the brain is damaged, these proteins are released into the extra-cellular fluid and the CSF. Increased CSF levels might therefore indicate (ir)reversible damage of certain cell structures [6-9]. Over the past 5 years, we have done a great deal of research into 3 brain-specific proteins:

- S-100, an acidic protein (M=21000). It exists as subtype S-100 a (subunits  $\alpha + \beta$ ) and subtype S-100 b (2 $\beta$  subunits). S-100 can be assayed by particle counting immunoassay (PACIA).
- NSE (M=90000) is the  $\gamma\gamma$  isoenzyme of enolase. Outside the CNS, NSE can be found in diverse cells of the neuro-endocrinological system. NSE can be assayed using a radio immunoassay (RIA) kit from Pharmacia.
- MBP (M=18500) is an alkaline protein and makes up about 30% of the protein in central and peripheral myelin. MBP can be assayed using a RIA kit from DSL [10]. As antigen human basic protein (whole molecule) is used and as antiserum polyclonal rabbit antihuman MBP.

### Diagnostic value

S-100, NSE and MBP are markers for glia cell damage, neuron damage and demyelination, respectively. Increased CSF S-100 levels can be observed after acute CNS damage, e.g. cerebrovascular accidents, viral infections and head injuries. The increase is related to the severity of the affection and the extent of the lesions. Increased NSE levels are often seen in association with traumatic, ischemic and infectious processes of the CNS as result of neuron damage. In chronic degenerative processes, low values are sometimes observed. MBP is increased particularly in patients with active demyelination. During acute relapse, MS patients show (strongly) elevated MBP values. In the remission phase, the levels are usually normal. Several authors have shown that the MBP level is an important parameter for the activity of the disease process [10-12]. The MBP concentration in MS is not related to the IgG index or intrathecal IgG production [10,11], but it is related to the IgM index and intrathecal IgM production [13] and to gadolinium-DTPA enhancement on MRI [14]. It has also been shown that in MS patients who have received a high dose of intravenous methylprednisolone, the increased MBP levels are reduced or normalized

and the decrease of MBP is related with decrease in IgM index [13] and with the clinical improvement of the patient [15]. We reported that the reference values of the above-mentioned proteins in children and adults are age-dependent [16]. Measuring brain-specific proteins in CSF can give an important indication about the affected brain compartment, provides information about the prognosis and monitors the effect of treatment. Obviously, the time of the lumbar puncture in relation to the onset of the disease is very important.

#### **Group 4: humoral immunology**

##### *Ig indices*

The absolute value of IgG concentration in CSF depends on many factors like: IgG concentration in serum, blood/CSF barrier function, age of the patient, volume of CSF extracted and finally local IgG synthesis in central nervous system. These influences restricted the diagnostic significance for detection of local IgG synthesis in CNS on the basis of increased absolute IgG concentration in CSF. This led very early on to the development of ratios, quotients to eliminate several of these individual variations in the single patient. The basic idea was the formation of CSF/serum concentration quotients [17]. The IgG index =  $\frac{\text{IgG CSF} \times \text{albumin serum}}{\text{IgG serum} \times \text{albumin CSF}}$ . The CSF/serum ratio of IgG eliminates the individual variation of serum IgG. By referring this IgG CSF/serum quotient to the albumin CSF/serum quotient, it is possible to eliminate the variation of the IgG quotient by the individual blood CSF barrier function too. There have been many approaches for the correlation of both these quotients to obtain a high sensitivity for the discrimination between a locally synthesized IgG fraction (in brain) and the blood derived IgG fraction in CSF. By empirical and theoretical aspects it became obvious that the relation between the CSF/serum quotients of two molecules of different size (e.g. IgG and albumin) can be described by a hyperbolic function [18]. This approach can be applied uniquely to CSF samples of ventricular, cisternal or lumbar origin from children or adults to identify local IgG synthesis in the central nervous system. It should be mentioned that a linear approach (index) is physiologically incorrect and must lead to a severe loss of accuracy in the range of the blood CSF barrier dysfunction, especially for larger

molecules like IgA or IgM. In figure 1 the (hyperbolic) relations between  $Q_{\text{alb}}$  and  $Q_{\text{Ig's}}$  for 361 neurological patients without immunological disturbances in CNS is presented. For evaluation of immunoglobulin indices, graphic representation of immunoglobulin quotients ( $Q_{\text{Ig's}}$ ) and barrier functions ( $Q_{\text{alb}}$ ) can also be used in a diagramm. It should be stressed that the calculation of these indices is not possible in case of haemorrhage.

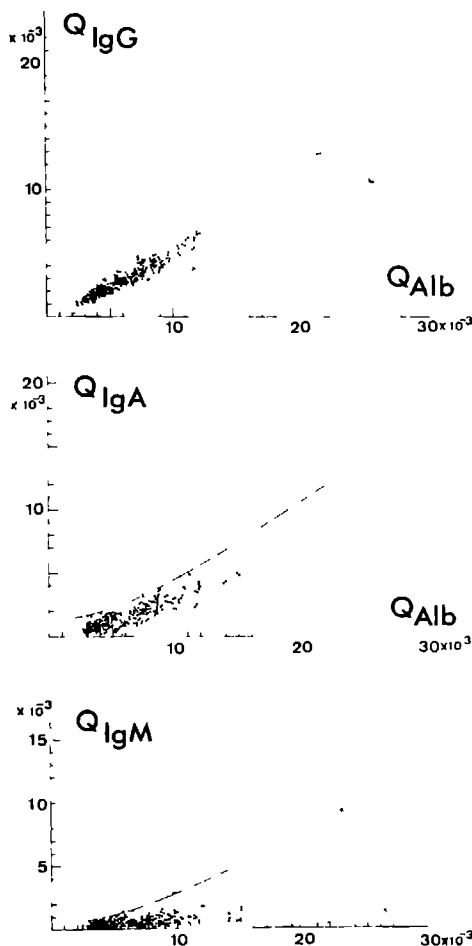


Figure 1. CSF/serum concentration quotients of the immunoglobulins G, A and M at the normal and pathological barrier with permeabilities up to  $Q_{\text{Alb}} = 30 \times 10^{-3}$  (double linear plot). The upper lines represent a hyperbolic function that is defined in the text. Data from  $n = 361$  patients were involved in each groups. (With permission of Reiber H. In: Protein transfer at the blood CSF barrier and the quantitation of the humoral immune response within the central nervous system. CCA 1987;163:319-328).

### Formulas for calculating intrathecal production of immunoglobulins

Besides the application of the Ig indices, various formulas have been reported over the past years for the calculation of intrathecally produced CSF IgG. Intrathecal IgG is total CSF IgG minus the transsudative IgG. The first formulas [19,20] were based on a linear relationship between Q Alb and Q IgG. More recent formulas make use of a hyperbolic [18] or exponential function [21]. The application of the latter two formulas led to a clear reduction in the number of false positive results in case of BCB disturbances, while the sensitivity is maintained. Soeverijn [22] compared Reiber's hyperbolic formula to 5 other formulas and showed that Reiber's formula produced the best accordance with the golden standard, the IEF. The calculation of intrathecal production of IgG and, in analogy to IgG, IgA and IgM has considerable diagnostic and prognostic potentials and can also be used for monitoring treatment.

### Diagnostic value

#### *IgG index*

An increased IgG index is observed in many neuroimmunological diseases, including infections. In more than 80% of patients with MS, increased IgG indices are found. It is not yet known about the antigen specificity of abnormal IgG in MS. There seems no relationship in MS between the severity or the activity of the disease and the intrathecal production of IgG [23,24]. Increased IgG indices are also found in about 30% of patients with chronic meningitis or encephalitis from various causes, such as bacteria, virus and protozoa and in diseases like: cerebral serum lupus erythematosus (SLE), polyradiculitis, sarcoidosis and chronic myelopathy.

#### *IgA index*

An increased IgA index is seldom found in MS patients (12%). In aseptic meningitis abnormalities are found in 40% and in herpes encephalitis in almost 100% as a result of virus-specific IgA antibody production [25]. In neurotuberculosis an increased IgA index is often accompanied by normal IgG and IgM indices. Determination of the dimer IgA index is more sensitive than the total IgA index [26].

### *IgM index*

IgM production is the first sign of a recent infection and of primary antigenic stimulation. IgG production occurs at a later (chronic) stage. Many publications have appeared on (abnormal) IgM indices in neurological diseases. In various infectious diseases, such as neurosyphilis, acute aseptic meningo-encephalitis, herpes encephalitis, HIV infection, an increased IgM index is measured in more than 50% of the cases [27]. Increased IgM indices are also seen in cerebral SLE [28] and in 30 to 60% of MS patients with a short disease duration [29,30]. Sharief [31] showed that an increase in the IgM index is usually associated with oligoclonal IgM bands on electrophoresis. This indicates an oligoclonal aspect of intrathecally produced IgM. According to this author the determination of oligoclonal IgM is more sensitive than the IgM index. The same author reported that cerebral IgM production is related to the activity of the disease in MS and that early detection of oligoclonal IgM in patients with acute isolated lesions of the brain stem and spinal cord has more predictive value for the development of MS than oligoclonal IgG [32]. IgM index determination is important for the early diagnosis of inflammatory neurological diseases and for monitoring the effect of therapy. A persistent IgM production in CNS indicates continuous antigenic stimulation. We can determine 4 Ig index profiles with respect to IgG, IgA and IgM for the differential diagnostics:

- IgG dominance with sporadically IgA and/or IgM increases, for example in MS and chronic HIV encephalitis.
- IgM dominance, for example in active neurosyphilis and in neuroborreliosis.
- IgG + IgA, for example in bacterial meningitis and neurotuberculosis.
- IgG + IgA + IgM, for example in mumps meningoencephalitis and in opportunistic infections.

### *Protein electrophoresis*

Over the past 30 years, CSF protein electrophoresis on agar or cellulose acetate has found an important application in the diagnosis of MS and infectious diseases of the CNS, particularly the detection of oligoclonal gamma bands. CSF oligoclonal banding indicates the presence of an intrathecal immune response. In the



meantime, new protein separation techniques have been developed to increase the sensitivity. IEF on agarose or polyacrylamide has been strongly recommended in recent years. Oligoclonal bands with help of IEF are observed much more frequent in comparison with conventional electrophoresis methods with agar or cellulose acetate. The latter methods therefore needed to be replaced by IEF with immunoblotting or immunofixation and the use of unconcentrated CSF and diluted serum. Staining can be performed by means of immuno-horseradish peroxidase labelling or with silver stain. It is necessary to perform parallel serum and CSF of the patient to establish CSF-specific oligoclonal bands. We perform IEF on agarose with Pharmalytes and stain after immunoblotting with immuno-horseradish peroxidase label, according to the method described by Thompson [33].

### *Antibody typing*

Oligoclonal gamma bands can be further characterized. The separated protein fractions are blotted on to a nitrocellulose membrane and labelled and detected by specific antibodies. In this way, for each immunoglobulin band, the isotype: IgG, IgA, IgM, kappa or lambda-free light chains, can be determined [30] as well as the possible identity of the antibody, e.g. anti-herpes, anti-borrelia or anti-HIV antibody.

### Diagnostic value

In MS, oligoclonal IgG bands restricted to the CSF are observed in more than 90% of the cases. In patients with chronic infection of the CNS (bacteria, virus, fungus) IgG oligoclonal bands are found in  $\pm$  40% of the cases like: herpes, varicella, toxoplasmosis, mumps, rubella, tuberculous bacilli, borrelia and aspergillus. These oligoclonal abnormalities are also found regularly in other inflammatory diseases, such as neurolupus. Oligoclonal bands may also be present in serum. If the CSF bands are a "mirror" pattern of the serum bands, the process is limited to the systemic compartment. If additional CSF bands are present, from a biochemical point of view, the immune process is extended to the CNS, e.g. in HIV infection, SSPE, neurosyphilis, neurosarcoidosis, neuro-Behcet's syndrome and neuroborreliosis. Finally, a paraprotein pattern can also be observed in which a monoclonal band pattern is

seen in both CSF and serum, due either to myeloma or so-called "benign" paraprotein. Figure 2 shows diagrams of CSF/serum couples in various neurological diseases using agarose IEF.

It is important to realize that local IgG production in CSF, established by means of an increased IgG index or oligoclonal bands, can be found many years after an adequate anti infection treatment e.g. neurosyphilis or neuroborreliosis. Oligoclonal IgG bands with antibody activity against the responsible agent are observed in the CSF in herpes encephalitis, meningoradiculitis (herpes zoster), SSPE, HIV infection, neurobrucellosis and tuberculous meningitis. Oligoclonal IgM bands are observed in MS (40%), neurosyphilis (22%), meningitis (44%) and encephalitis (22%). Oligoclonal IgM disappears again rapidly, in contrast with oligoclonal IgG. The presence of oligoclonal free kappa and lambda chains in CSF is a sensitive indication for recent antigenic immune response within the CNS, comparable with IgM. According to Sindic et al. [30] the detection of oligoclonal free kappa chains in CSF is the best test to support the diagnosis of MS. In addition, free light chains can also be found in the CSF in inflammatory diseases of the CNS.

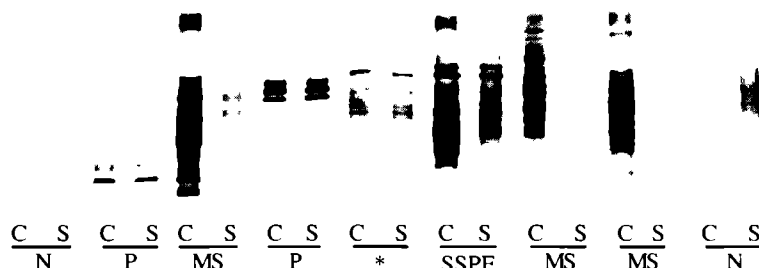


Figure 2. IEF couples (CSF + serum) in 9 neurological patients. Method: agarose IEF with unconcentrated CSF and diluted serum, overblotting on to nitrocellulose membrane and immunodetection of IgG by means of double antibody coupling with horseradish peroxidase as staining method. C = CSF, S = serum, N = normal, P = paraproteinemia, MS = multiple sclerosis, \* = systemic immunopathology ("mirror" pattern), SSPE = subacute sclerotic panencephalitis. (Received from Thompson E. Institute of Neurology, Queen Square, London, with permission).

### *Complement components C3 and C4*

The complement system activates various inflammatory reactions by means of

its component products and participates in immune regulation. An increase in C3 and C4 components indicates activation of the complement system. Increased C3 and C4 consumption may be seen in association with immune complex formation. Macrophages, lymphocytes and astrocytes in the brain can produce these components. Intrathecal production of C3 and C4 components can be established by means of the determination of C3 and C4 indices, in analogy to the IgG index. An increased C4 index indicates intrathecal production, while a decreased index indicates intrathecal consumption.

#### Diagnostic value

Intrathecal C3 and C4 production has been established in aseptic meningitis [34]. In 37 patients infected with HIV 1 virus, intrathecal C4 production could be measured in 80% [35]. C4 production is correlated with IgG production. It is possible that C4 production in combination with many antibodies can neutralize the virus [36]. C3 and C4 production was also demonstrated in MS patients during exacerbation [37]. In 12 patients with diffuse CNS SLE, we found a significantly increased C4 index which was correlated with an increased IgM index [28]. Determination of C3 and C4 indices may be important for (differential) diagnosis and to measure the activity of auto-immune diseases and/or inflammatory diseases of the CNS.

#### **Group 5: cellular immunology**

Lymphocytes (subpopulations) are involved in the cellular and humoral immune response. Blood lymphocytes reflect the systemic immune response and CSF lymphocytes the immune response within the brain compartment. It is assumed that only 'activated lymphocytes' can pass the BCB. With help of new methods, it has become possible to evaluate lymphocyte subsets in CSF which contains a low or normal number of cells. We have developed a sensitive micro-fluorescence method [38]. Flow cytometry can also be used for these tests [39]. At our laboratory, the following CSF and blood lymphocyte subsets are assayed routinely: CD3: T cell, CD4: helper/inducer T cell, CD8: suppressor/cytotoxic T cell, HLA/DR: MHC class II antigen, CD20: B cell. Lymphocyte subsets have been studied in CSF and blood in

various neurological diseases, including infections of the CNS [40,41]. Abnormalities were found in the relative proportions of lymphocyte subsets in CSF. Also in MS patients studies are performed in order to determine the cellular immunity involvement of the brain. Decreased percentages of CD8 have been reported in CSF in these patients [38,42,43]. Other studies could not confirm this [39,44].

### Diagnostic value

Lymphocyte subset examination might provide information on the immune (dys)regulation of neuro-immunological diseases and might monitor the effect of treatment.

### **Group 6: brain metastases and tumour markers**

In 32% of cancer patients, intracranial metastases are detected at autopsy [45]. Although CT scanning is an accurate and sensitive diagnostic method, in the case of, for example, leptomeningeal metastases, positive findings are only moderate ( $\pm$  34% [46]). MRI is now more sensitive in detecting such metastases. Cytological CSF investigation is certainly an important diagnostic tool, but even with this approach, malignant cells are only found in 26% of all the cases with proven metastatic tumours [1]. Over the past few years, studies have been published on the determination of tumour markers in the CSF of patients with brain metastases. Research has concentrated particularly on carcino embryonic antigen (CEA) because tumours which produce CEA show a strong tendency to metastasize to the brain compartment [47]. The normal CEA level in CSF is very low ( $< 5$  ng/l). Reiber et al. [48] reported that in analogy with the IgG index, CEA transport from the blood to the CSF/brain compartment depends on the function of the BCB and that the CSF CEA concentration depends on the blood concentration and the BCB function. An increased CSF CEA level as a result of an increased serum level and/or disturbed BCB can be distinguished from an increased CSF level due to intrathecal synthesis by means of the CEA index. By definition, a CEA index  $> 1.0$  indicates an intrathecal synthesis. However, it is not always possible to measure the CEA concentration in normal CSF even using a sensitive CEA assay. In cooperation with our laboratory, a

CSF "CEA-sensitive" enzyme immunoassay is developed by ELIAS, Medizin Technik GmbH, Freiburg, Germany. Besides CEA,  $\beta_2$ -microglobulin and  $\beta$ -glucuronidase are also mentioned in literature as valuable markers for the detection of CNS metastasis of leukaemia, lymphoma and solid tumours [49].

### Diagnostic value

In 16 out of 18 patients ( $\pm 90\%$ ) with meningeal metastases and in 14 out of 30 patients ( $\pm 45\%$ ) with parenchymal metastases, Jacobi [50] measured intrathecal CEA production. The chance of detecting parenchymal metastases by means of CSF CEA analysis decreases as the distance between the affected brain area and the ventricular system increases. In 55 patients with a primary brain tumour, intrathecal CEA production was observed in only 10%. In patients with a CEA producing tumour, but without brain metastases, no increase in the CEA index was found. We observed an increased CEA index in 6 patients with proven brain metastases (see Figure 3).

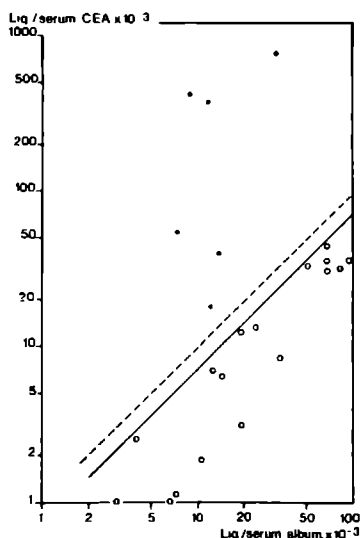


Figure 3. Double logarithmic presentation of the relationship between CSF serum albumin ratio (Q alb) and CSF serum CEA ratio (Q CEA) in 17 neurological control patients without metastases (open circles) and 6 patients with proven metastases in the CNS (closed circles). The discrimination lines have the function  $Q\ CEA = 0.7\ Q\ alb$  (solid line) and  $Q\ CEA = 1.0\ Q\ alb$  (dotted line). (With permission from Reiber H. Sensitive quantitation of CEA in CSF and its barrier-dependent differentiation CCA 1986;156:259.270 with minor alterations)

In addition to CEA, Ongerboer de Visser et al. [49] investigated the tumour markers  $\beta_2$ -microglobulin and  $\beta$  glucuronidase in CSF. They encountered an increase in  $\beta_2$ -microglobulin level particularly in leptomeningeal metastases from haematological tumours. However, raised  $\beta_2$ -microglobulin levels have also been found in association with infections within the CNS. An increase in the  $\beta$ -glucuronidase level forms an indication of leptomeningeal metastases. The analysis of tumour markers in CSF can support the diagnosis of brain metastases and can monitor the effect of oncological therapy.

#### Group 7: neurotransmitters

A great deal has been published on the relationship between CSF neurotransmitters (metabolites) and the metabolism of the CNS. It has been established that homovanillic acid (HVA) is a metabolite of the dopaminergic system, 5-hydroxyindole acetic acid (5-HIAA) of the serotonergic system and 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG) of the noradrenergic system. Vanilmandelic acid (VMA) is not found in brain tissue. Factors such as ventriculospinal gradients, 24-hour rhythms, physical activity and age influence the basal CSF values. The ventriculo/lumbar ratio for the concentration of HVA, 5HIAA and MOPEG is 10, 4.5 and 1, respectively. Our studies on young children have shown that the 5HIAA and HVA levels are much higher than those found in older children and adults, which might indicate a higher turnover or alteration in clearance rate. An important study was published recently by Wester et al. [51]. They reported that ventricular CSF dopamine and HVA originate almost exclusively from the striatum, CSF MOPEG correlates with MOPEG in the hypothalamus, temporal cortex and pons, and CSF 5HIAA correlates with 5HIAA in the thalamus, hypothalamus and cortex. There is no relationship between CSF gamma aminobutyric acid (GABA) and specific areas of the brain. The spinal cord makes an additional contribution to neurotransmitters (metabolites) in the lumbar CSF. According to the above mentioned authors, CSF monoamine (metabolites) levels are a good indication of the central monoaminergic activity. High pressure liquid chromatography (HPLC) in combination with electrochemical detection is a suitable method for analysing monoamine (metabolites).

We have shown that GABA can be measured adequately with an amino acid analyzer in combination with fluorimetric detection after postcolumn derivatisation with orthophtaaldehyde [52].

### Diagnostic value

In patients with Parkinson's disease, low HVA, 5HIAA and GABA levels are found. Opinions differ with regard to the relationship between CSF HVA and the severity of the movement disorder. CSF values may help to distinguish between dopa-resistant and dopa-sensitive patients [53]. The lowest CSF 5HIAA concentrations are found in patients with Parkinson's disease who are suffering from severe depression [54]. L-Dopa administration increases CSF HVA, but not 5HIAA and MOPEG. Decreased GABA and HVA concentrations are found in patients with Huntington's disease. Medication to increase the GABA activity are not very successful. In tardive dyskinesia, no monoamine abnormalities are found in the CSF. Low levels of HVA, 5HIAA and GABA are found in patients with Alzheimer's disease. Decreased 5HIAA concentrations are also found in dementia patients and the concentration is lowest in multi-infarct dementia [55]. Low levels of 5HIAA may also be found in patients with vital depression. As yet, CSF neurotransmitter determination has little significance in the diagnosis of a special neurological disease. The investigation may contribute to evaluating the severity or prognosis of the disease, or to regulating and monitoring medication.

### *CSF sampling*

In view of the ventricular/lumbar gradient of these metabolites, we standardize the volume of the collected CSF. The first 8 ml of the CSF (tube 1) are used for routine tests, the next 3 ml (tube 2) are used for neurotransmitter determination.

### **Group 8: vitamins**

For the correct functioning of the CNS, vitamins B12 and folic acid must be imported from the blood. Both these vitamins play a role in the formation of methionine which can be further converted into S-adenosylmethionine. The latter

compound, as methyl donor, plays an important role in myelin synthesis. It has been suggested that a defective metabolism of the methyltransfer pathway with a reduced supply of methyl-groups and low S-adenosylmethionine may be a cause of demyelination [56,57]. It is remarkable that the folate concentration in CSF is maintained at a considerably higher level than the concentration in blood through an active carrier system in the choroid plexus. No such system is present for vitamin B12. Therefore, the vitamin B12 concentration in CSF is more than 10 times lower than the concentration in blood, which makes it very difficult to measure in CSF. Nevertheless, the concentration can be measured using 5 ml CSF which has been concentrated 10 times. Comparisons between the concentrations found in CSF and blood show that there is a clear relationship between them for both vitamins [58,59].

#### Diagnostic value

So far, there are little clinical applications for vitamin assays in CSF in individual patients. Both decreased CSF vitamin B12 and CSF/serum vitamin B12 ratio are demonstrated in a group of 42 patients with dementia, who could not be assigned to a definite traditional diagnostic group [60]. For groups of patients with MS and patients with Alzheimer's type dementia, we reported significantly decreased CSF vitamin B12 values [58]. In a subsequent study we have shown that there is a relationship between corticosteroid treatment and decreased vitamin B12 and folic acid concentrations in the CSF of MS patients [61].

#### **Group 9: metabolic investigation**

##### **Glucose, lactate and pyruvate**

For metabolic investigation of CSF (glucose, lactate and pyruvate), it is necessary to fix (a portion of) the CSF immediately to prevent any further metabolism of glucose and pyruvate.

##### **Glucose**

The CSF glucose concentration is strongly dependent on the blood glucose concentration. Therefore, simultaneous blood and CSF analyses must be performed.



The CSF/blood glucose ratio is about 0.65. In a previous study on children aged 3 to 16 years, we were not able to establish an age-dependent ratio for glucose [62]. Transport of glucose over the BCB is carrier-dependent.

### Diagnostic value

An increased CSF glucose concentration has no diagnostic value. A decreased glucose level (after the exclusion of hypoglycemia) occurs particularly in acute purulent meningitis, tuberculous meningitis, meningitis carcinomatosis, fungal infections, meningeal sarcoidosis and cysticercosis. It is possible that the glucose level remains low 2 weeks after adequate treatment, while pleocytosis and the increased albumin ratio content have normalized. A study by Donald [63] on children with either aseptic meningitis (n=97) or bacterial meningitis (n=119), showed that a CSF blood glucose ratio of  $< 0.40$  and/or a CSF glucose level of  $< 2.2$  mmol/l are suspicious of bacterial meningitis. In the case of viral meningitis, the CSF glucose level is normal, with the exception of mumps meningitis and herpes simplex encephalitis ( $\pm 25\%$  abnormal). A low glucose level in meningitis is probably caused by increased anaerobic glycolysis (also by bacteria) and by inhibition of the glucose transport over the BCB. A low glucose level can also occur in association with cerebral haemorrhage, as a result of anaerobic glycolysis by erythrocytes and hypoxia.

### **Lactate, pyruvate and L/P ratio**

It is generally accepted that the CSF lactate concentration is a good reflection of the brain lactate concentration. The CSF lactate concentration is hardly influenced by the (raised) blood lactate concentration, except in the case of obvious BCB disturbances [64]. The CSF L/P ratio reflects the redox state of the brain. In case of increased cerebral glycolysis (hypoxia, ischemia), the CSF lactate and L/P ratio increase. The increase in L/P ratio in association with hypoxia is caused by an increase in the conversion of pyruvate into lactate as a result of an increase in the NADH/NAD<sup>+</sup> ratio.

### Diagnostic value

The CSF lactate concentration is an important parameter for the metabolic status of the brain and has frequently been the subject of study in various neurological diseases. For the differential diagnosis of meningitis, a (strongly) raised lactate level occurs particularly in untreated bacterial or fungal infections, contrary to viral infections. The level of the increased CSF lactate in infections generally is an indication for the severity and the prognosis, and a decrease in the lactate level appears a measure for the efficacy of the treatment. In patients with bacterial meningitis, Kömel [65] reported a relationship between the number of granulocytes/monocytes in the CSF and the increase in lactate concentration. Hypoxia in the affected tissue, decreased blood flow and anaerobic glucose consumption by leukocytes, are probably responsible for the raised lactate level. In the case of cerebrovascular disease, the CSF lactate concentration also is an important measure of the severity and prognosis of the disease. Lamers examined the lactate concentration in 302 patients with an acute cerebrovascular disease and found a clear relationship between the lactate concentration and the severity of the stroke (see Figure 4) [66].

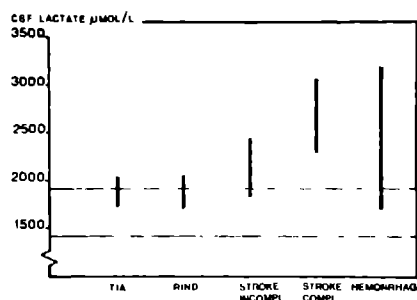


Figure 4. CSF lactate values (p10-p90) in 5 types of cerebrovascular accident. Transient ischemic attack = TIA; reversible ischemic neurological deficits = RIND

Busse [67] found a strong relation between CSF lactate and ischemic cerebral oedema using CT scanning in cerebral infarcted patients; none of the patients with a lactate value of  $> 4000 \mu\text{mol/l}$  survived. Besides hypoxia, other factors can be responsible for an increased CSF lactate, such as vitamin B1 deficiency or heavy metal

intoxication in which the enzymatic conversion of pyruvate into the Krebs cycle is inhibited. In this case, the L/P ratio is usually normal. In addition, an increase in the lactate level can occur in case of haemorrhage as a result of anaerobic glycolysis by erythrocytes and in most cerebral tumours. If all the above mentioned causes have been excluded, an increased CSF lactate level is very suspicious for a (congenital) metabolic defect in the mitochondrial transition of pyruvate in the brain, as is the case in the mitochondrial encephalomyopathies (e.g. Melas, Merff).

### **Amino acids, purines, pyrimidines and ketones**

Patients with congenital metabolic disturbances commonly suffer from neurological symptoms. In these cases it is likely that the metabolism of the brain will also be disturbed as a result of a local metabolic defect and/or abnormal substrate availability. Supplementary investigation of the CSF (in addition to plasma and/or urine) for metabolites can possibly elucidate pathophysiology of the brain metabolism in this group of patients. A few publications have appeared in the literature on metabolic CSF analysis in the above-mentioned group of patients [68-70]. It can be expected that in children with, for example, unexplained mental retardation or epilepsy, disturbances in the brain metabolism may be the underlying cause. Unfortunately, there are very few reliable age-dependent reference values for these metabolites in CSF, particularly in children. Therefore several years ago, we started an extensive study to measure metabolic products in the CSF and plasma of more than 1000 children who underwent a diagnostic lumbar puncture on the suspicion of an infectious disease or an other neurological disorder. The study was aimed at amino acids, purines, pyrimidines and fuel-related substrates (glucose, alanine and ketones) after prolonged fasting. Age-dependent reference values for these products in CSF have now been established and published [52,62,70]. Follow-up studies on selected neurological patient groups are in progress. Clear elevations in CSF of argininosuccinic acid, pseudouridine and uridine were observed in 3 children with the late onset form of argininosuccinic aciduria [72]. In 10 children with chronic renal failure a marked increase of pseudouridine and cytidine was demonstrated in CSF [73]. Recently in 7 patients with biotinidase deficiency increased values of CSF lactate

and 3-hydroxy isovaleric acid were observed. The values in CSF were higher than in plasma [74]. Abnormalities of amino acids levels in CSF in association with other neurological disorders besides metabolic disturbances are also reported [75].

### **Recommended CSF investigations at a general hospital laboratory**

A general hospital laboratory should be able to perform the following routine CSF analyses for neurological diagnosis:

- Leukocyte and erythrocyte number.
- Leukocyte differentiation.
- Total protein content or (better) albumin ratio.
- Lactate, glucose.
- Detection of oligoclonal IgG bands. The recommended method is IEF with unconcentrated CSF and diluted serum, in combination with immunoblotting or immunofixation.
- IgG index as option: the determination of the IgG index cannot replace IEF, not even as prescreening.

All the other CSF analyses are so specialized and the frequency is so low that they can be left to a selected number of specialized laboratories. During evenings, nights and weekends, only total protein, cells and glucose should be determined.

Table 2 shows the CSF and serum reference values at our laboratory and the necessary quantities of CSF.

Table 2. Reference values (p5-p95) of CSF and serum components in adults determined at our laboratory. The necessary quantity of CSF is mentioned per group.

group	component	CSF ml	unit	CSF reference value	serum reference value	index/ratio
1	leukocytes	1.0	/ $\mu$ l	<5		
	haemoglobin		$\mu$ mol/l	<0.025		
	bilirubin		$\mu$ mol/l	<0.08		
	total protein	0.8	mg/l	180-580	60-80 g/l	
	albumin (ratio)		mg/l	130-380	35-55 g/l	1.7-9.0 (*)
	LD		U/l	8-30	<330	( $\times 10^{-3}$ =ratio)
2	calcium	0.6	mmol/l	1.02-1.18	2.20-2.60	0.5-0.8 (=ratio)
	phosphate		mmol/l	0.40-0.55	0.80-1.30	
	potassium		mmol/l	2.7-3.1	3.4-4.6	
	sodium		mmol/l	144-152	137-144	
	chloride		mmol/l	120-128	98-107	
3	MBP	0.5	$\mu$ g/l	0.2-1.5	2.0-7.0	
	S-100		$\mu$ g/l	1.4-4.6		
	NSE		$\mu$ g/l	3.7-12.6		
4	IgG	0.5	mg/l	11-38	7.4-16.2 g/l	0.36-0.60
	IgA		mg/l	0.6-4.0	0.8-3.8 g/l	0.10-0.41
	IgM		mg/l	0.1-0.4	0.3-2.0 g/l	0.01-0.07
	IEF	0.5				
	C3 complement	0.4	mg/l	0.3-5.8	580-1200	0.27-0.75
	C4 complement		mg/l	0.4-2.8	160-460	0.12-1.56
5	lymphocyte subsets	>10	%			
6	CEA	2.5	$\mu$ g/l	<0.010	<5.0	<0.7
	$\beta$ 2-microglobulin		mg/l	0.8-3.0	1.0-4.0	
	$\beta$ -glucuronidase		U/l	10-27	75-265	
7	HVA	2.0	nmol/l	100-400		
	5HIAA		nmol/l	40-170		
	MOPEG		nmol/l	25-55		
	GABA		nmol/l	50-610		
8	folic acid	5.0	nmol/l	14-42	5.5-19	1.3-4.3
	vitamin B12		pmol/l	2.1-22.9	142-541	1.0-6.6 ( $\times 10^{-2}$ =ratio)
9	glucose	1.0	mmol/l	2.5-3.7	4.0-5.5	0.5-0.8 (=ratio)
	lactaat		$\mu$ mol/l	1380-1900	700-1800	
	pyruvaat		$\mu$ mol/l	100-145	41-68	
	L/P ratio		-	11.7-16.5		
	amino acids	1.0				
	purines/pyrimidines					

(\*) = age dependent reference values apply to children.

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## **CHAPTER 3, section 1**

### **THE CONCENTRATION OF BLOOD COMPONENTS RELATED TO FUEL METABOLISM DURING PROLONGED FASTING IN CHILDREN**

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## SUMMARY

In order to study the relationship between sex, age and glucose, and the concentrations of various fuel related blood substrates in children during prolonged fasting, we have selected data of fasting procedures in 13 control children aged 3-5 yr, fasted 24 h, and 58 control children aged 6-15 yr, fasted 40 h.

Compared to the blood results after overnight fast, glucose is decreased, and lactate, pyruvate, ketones and non-esterified fatty acids (NEFA's) are all clearly increased at the end of fast. The concentrations of alanine and triglycerides remain unchanged. The relation with sex, age and glucose has only been analyzed in the older children group. A sex-dependency is indicated for the ketones. Ketones are negatively related with age. NEFA's, pyruvate and alanine are not age-related, whereas glucose, lactate and triglycerides are moderately age-dependent. Ketones are negatively related with glucose, whereas pyruvate, NEFA's and triglycerides are not glucose-related. Lactate and alanine are weakly related to glucose.

The data demonstrate diminished glucose homeostasis and increased ketogenesis in younger children compared to older ones during prolonged fasting.

## INTRODUCTION

Adaptation to fasting is a complex of interrelated mechanisms, involving regulation of substrate mobilization, interconversion and utilization. Monitoring this transition has been recognized as a particularly useful tool in the differential diagnosis of childhood hypoglycemia and other disturbances in energy metabolism [1-10]. There are many publications dealing with fasting studies in children [2,6,8,11]. Metabolic and hormonal responses have been measured under various fasting conditions. From recent studies it became clear that substrate responses in children after fasting are age-dependent [2,5,7,9,10,12-14]. The relationship between the blood glucose concentration and the concentrations of other energy-yielding blood substrates during fasting has also been described [2,5-10,13,14]. However, fuel related components in blood during fasting have not been systematically investigated and therefore the results from the various studies are only partly comparable. Regularly, the number of children in the fasting experiments is low and the age and sex distribution is inadequate. Moreover, the period of fasting with older children is often too short and the spectrum of the investigated substrates is not complete with respect to fuel metabolism. Therefore, we collected data of prolonged fasting procedures in children. These data met the following conditions: a sufficient number of children to study age and sex effects and the relation with blood glucose; fasting periods adjusted to age; biochemical analysis of the relevant circulating blood substrates with respect to fuel metabolism.

In this paper, data are presented on blood glucose, lactata, pyruvate, acetoacetate,  $\beta$ -hydroxybutyrate, plasma non-esterified fatty acids (NEFA's) and serum alanine, triglycerides and cholesterol concentrations.

The purpose of our investigation was, on the one hand, to study fuel related substrates in children and the relationship of sex, age and glucose with these substrates during prolonged fasting, and on the other hand, to obtain reference values related to age after a distinct fasting period (24 h in children aged 3-5 yr, 40 h in children aged 6-15 yr).

## SUBJECTS AND METHODS

### Subjects

Over a period of 5 yr a total of 71 children, 25 girls and 46 boys, ranging in age from 3-15 yr, were included in this study. These children were observed clinically because of intermittent behavioral problems and/or unexplained seizures, where the possibility of a hypoglycemia disorder was considered or had to be ruled out. They were classified retrospectively as suitable reference subjects because of lack of evidence for endocrine or metabolic abnormalities. Appropriate studies ruled out immunologic and chronic infectious diseases, deficiencies and disorders caused by toxic agents. The children were divided into two groups according to age (table 1) and fasting period. Informed consent of the parents and of the older children themselves was obtained before the investigation.

Table 1: Age characteristics of the subjects.

		Number			Age (mean $\pm$ SD)		
		Boys	Girls	Total	Boys	Girls	Total
Total group	3-15 yr	46	25	71	8.7 $\pm$ 3.4	10.5 $\pm$ 3.2	9.3 $\pm$ 3.4
Group I	3-5 yr	13	-	13	4.8 $\pm$ 0.9		4.8 $\pm$ 0.9
Group II	6-15 yr	33	25	58	10.2 $\pm$ 2.8	10.8 $\pm$ 2.8	10.5 $\pm$ 2.8

### Fasting test procedure

The subjects were on a normal hospital diet for at least 72 h before the test. Fasting was always started after a last meal at 6 p.m. Only drinking of water was allowed during the fast. For group I, blood was sampled after 15 h and at the end of the 24-h fast, for group II after 14 h and at the end of the 40-h fast.

### Biochemical and statistical procedures

Biochemical methods have been reported previously [14].

Except for the ketone bodies, statistical analysis was performed within the previously reported linear model [14], which gives a satisfactory fit to the data both

for the blood concentrations at 14-h and at 40-h of fasting. Due to the rather constant median level of the ketones in children aged 6-11 yr, it was necessary to modify the statistical model for the blood concentrations of the ketones at 40-h fast. Therefore, for these ketone values, instead of the standard regression model with age, we fitted a two-phase regression model using the technique described by Hinkley [15].

## RESULTS

In table II we present the median concentrations of the blood components at overnight fast and at the end of the fast for groups I and II.

Group I (age 3-5 yr): in comparison with the median overnight fasting blood concentrations, glucose is decreased, and lactate, pyruvate,  $\beta$ -hydroxybutyrate, acetoacetate (ratio  $\beta$ -hydroxybutyrate/acetoacetate, total ketone bodies) and NEFA's are all clearly increased at the end of fast. The ratio lactate/pyruvate and the concentrations of alanine, triglycerides and cholesterol remain unchanged.

Group II (age 6-15 yr): the same phenomena as in group I are found for the various blood components in group II.

Table II: Median blood values after overnight (14 h) and prolonged fast (respectively 24 h and 40 h).

Blood parameter	Unit	Boys aged 3-5 yr (n=13)		Boys and girls aged 6-15 yr (n=58)			
				Boys (n=33)		Girls (n=25)	
		14 h	24 h	14 h	40 h	14 h	40 h
Glucose	mmol/l	4.3	3.5	4.4	3.4	4.3	3.6
Lactate	$\mu$ mol/l	860	1750	840	1640	840	1720
Pyruvate	$\mu$ mol/l	97	167	87	177	84	200
Ratio L/P		9.8	11.1	9.0	9.6	9.0	9.2
Alanine	$\mu$ mol/l	284	292	332	345	362	354
$\beta$ -OH-butyrate	$\mu$ mol/l	321	2068	117	3687	100	2836
Acetoacetate	$\mu$ mol/l	115	551	93	723	78	600
Ratio $\beta$ -OH-B/AcAc		2.8	3.7	1.5	4.6	1.7	4.4
Total ketone bodies	$\mu$ mol/l	397	2561	242	4444	170	3450
NEFA's	$\mu$ mol/l	990	1975	700	1780	675	1775
Triglycerides	mmol/l	0.89	0.84	0.62	0.69	0.84	0.83
Cholesterol	mmol/l	4.2	4.6	4.2	4.6	4.1	4.3

## Relation between sex and age and blood concentrations at the end of the fasting period

While the number of children and the age range in group I is small, statistical analyses of the relation of sex, age and glucose with blood concentrations of the various biochemical parameters were not considered. In the older children group II, however, we have evaluated these matters. Within the statistical model referred to (see statistical procedures), the effects of sex and age on blood levels after 40-h fast were tested for their significance.

### Sex

A moderate sex-dependency is indicated only for alanine and total ketone bodies ( $p = 0.06$  and  $0.03$ , respectively). The median blood ketone level for boys at 40-h fast is 1.18 times the level for girls, adjusted for age. The ratio  $\beta$ -hydroxybutyrate/acetoacetate is significantly sex related ( $p = 0.02$ ).

### Age

Age effects, while adjusting for possible sex differences, are clearly ( $p < 0.001$ ) demonstrated for  $\beta$ -hydroxybutyrate, acetoacetate, (ratio  $\beta$ -hydroxybutyrate/acetoacetate, total ketone bodies). A moderate ( $0.05 < p < 0.10$ ) age-dependency is indicated for glucose, lactate and triglycerides. For other components any age-dependency is not apparent. The ketones and the ratio  $\beta$ -hydroxybutyrate/acetoacetate are negatively correlated with age.

In table III, the mean value and the standard deviation are presented for the various blood components in group II. Within the statistical model used, estimates  $\hat{p}_{2.5}$ ,  $\hat{p}_{50}$  and  $\hat{p}_{97.5}$  are given for respectively the 2.5, 50 and 97.5 percentiles. For the variables that show clear age-dependency, estimated percentile values are presented for 6-, 9- (12- when ketones are concerned) and 15-yr-old children. For illustrative purposes, individual values together with the estimated percentiles are presented for ketone bodies in figure 1.

Table III: Blood values after 40-h fast for 6- to 15-yr-old children.

Blood parameter	Unit	Mean	SD	Age	P2.5 <sup>a</sup>	P50 <sup>a</sup>	P97.5 <sup>a</sup>
Glucose	mmol/l	3.44	0.44	6-15	2.64	3.41	4.41
Lactate	μmol/l	1795	561	6-15	962	1720	3070
Pyruvate	μmol/l	190	49	6-15	108	183	311
Ratio L/P	-	9.55	1.96	6-15	6.31	9.36	13.90
Alanine	μmol/l	357	72	6-15	240	351	513
β-OH-butyrate	μmol/l	3151	1384	6-11	1360	3480	8900
				12	1010	2570	6580
				15	490	1250	3180
Acetoacetate	μmol/l	661	250	6-11	333	710	1520
				12	278	594	1270
				15	181	386	814
Ratio β-OH-B/AcAc	-	4.79	1.78	6	3.18	5.64	10.00
				12	2.30	4.08	7.24
				15	1.96	3.47	6.16
Total ketone bodies	μmol/l	3812	1555	6-11	1770	4200	9980
				12	1350	3200	7600
				15	700	1660	3950
NEFA's	μmol/l	1779	615	6-15	779	1670	3560
Triglycerides	mmol/l	0.78	0.23	6-15	0.40	0.74	1.36
Cholesterol	mmol/l	4.60	1.04	6-15	2.94	4.50	6.88

<sup>a</sup> Estimates within the lognormal model

### Relation between the concentration of glucose and various blood substrates at the end of the fasting period

For children of the same age, the concentrations of β-hydroxybutyrate, acetoacetate (and total ketone bodies) are negatively correlated with glucose, respectively,  $r = -0.53$ ,  $r = -0.39$ ,  $r = 0.53$  (all  $p$  values  $< 0.05$ ). No significant correlation is found for pyruvate, NEFA's, triglycerides and cholesterol. Lactate (ratio lactate/pyruvate), alanine and ratio β-hydroxybutyrate/acetoacetate are all moderately correlated with glucose.

### Relation between the concentrations of ketones and serum alanine at the end of the fasting period

In view of the discussion about the supposed non-hormonal regulation of muscle alanine release by ketone bodies [16,17], we have tested also the correlation between ketones and alanine in blood at the end of fast. The correlation coefficient



between alanine and total ketone bodies was  $-0.28$  ( $p < 0.05$ ). Controls for age as well as for glucose give similar results.

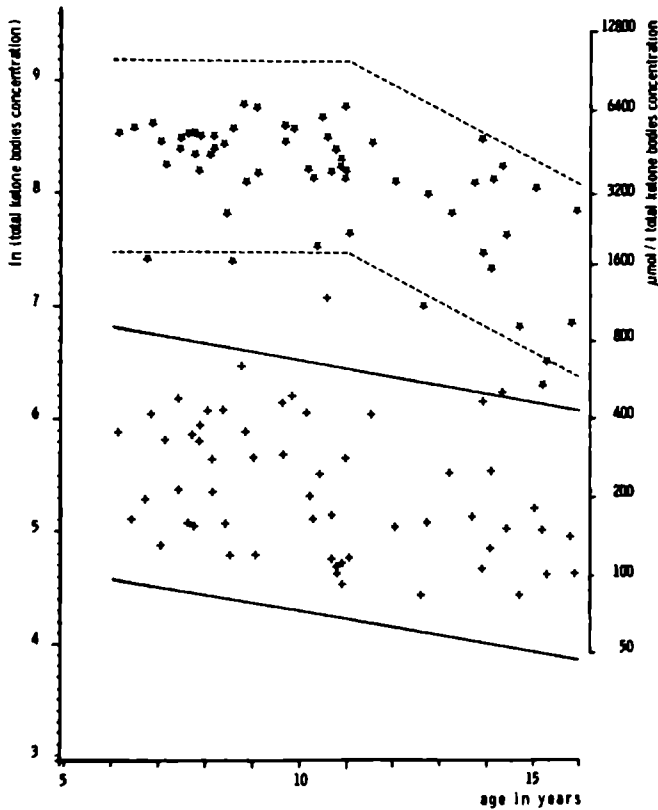


Figure 1: Total ketone bodies concentrations after 14-h (+) and 40-h (\*) fast.

## DISCUSSION

It is known that hormones play an important role in regulating blood glucose concentration and other fuel related substrates during fasting. The hypoglycemic effects of insulin are counter-regulated by the actions of adrenocorticotrophic hormone (ACTH), cortisol, glucagon, epinephrine and growth hormone. The net effect of these hormones during fasting is to stabilize the blood glucose level and to provide free fatty

acids and ketones as a source of energy. Hormonal responses during fasting have been studied in children and adults [2,10,13,18]. Differences were observed between young and old children [10]. The differences in the fasting responses of fuel related blood substrates (glucose, alanine, NEFA's and ketones) were even more striking. Therefore, we have studied blood substrate responses during fasting periods of 24 h in young children (n = 13) and of 40 h in old children (n = 58).

In our study, the pattern of changes in concentration of glucose, NEFA's and ketones during fasting agrees well with other observations of fasting experiments [2,3,5-11,13,18]. The concentrations of lactate and pyruvate clearly increase in the period from overnight fast to the end of the fast. The same phenomenon was also observed by Haymond et al [13] in children as distinct from adults. This increase can possibly be explained by an inhibition of the pyruvate dehydrogenase complex by both an increased mitochondrial acetyl-CoA level from ketone-utilization and an increased mitochondrial NADH concentration [20], indicated by the increased ratio  $\beta$ -hydroxybutyrate/acetoacetate at the end of fast in our study; a phenomenon which has been described also by Huth et al [21]. The ratio lactate/pyruvate as an index of the cytosolic redox state remains stable. The blood alanine concentration does not change in our study from overnight fast to the end of fast. Other observations showed that blood alanine decreases very strongly during the first 12-16 h postprandially, while after prolonged fasting, the blood alanine level decreases at a much lower rate [10,13] or remains relatively stable [8]. In literature, no explanation was given for this alanine effect with respect to fasting time.

The relationship between blood parameters and sex, age and glucose concentration in the group of children between 6 and 15 yr of age was also studied.

### *Sex effect*

A sex-dependency was found for ketones after 40-h fast, including the ratio  $\beta$ -hydroxybutyrate/acetoacetate. There is a tendency that after prolonged fasting, in contrast to overnight fasting, girls develop a lower level of ketone bodies than boys. Data in the literature about sex-dependency in fasting children are very scarce. Only in adults, Haymond et al [13] studied differences in fasting substrate responses

between men and women. Glucose, alanine and ketone bodies responses were significantly different (higher) in women compared to men [13]. This is not found for children in our study.

### *Age effect*

The glucose response after 24-h fasting in both children groups is different; younger children show lower glucose concentrations than older ones. This is in agreement with observations from other studies [5,7,9,10]. However, in group II, at the end of fast (40 h) there is no clear age-dependency for glucose. The fat-derived ketones and NEFA's increase rapidly in the period between overnight fast and the end of fast, and a clear age-dependency is found for ketones (fig 1). This finding agrees well with other observations [7,9]. The two-phase regression model fits the ketone data much better than the standard one-phase regression model. This might indicate that children in the age range 6-11 yr reach a ketone level after 40-h fasting where production and utilization of the ketones are in balance (fig 1). Another possibility could be that a feedback mechanism stabilizes the ketone production. Neither the concentration of NEFA's nor the serum alanine level show any age relation at 40-h fast. Chaussain et al [5] found a clear age-dependency for alanine in children after 24-h fasting, and we found the same phenomenon after 14-h fasting [14]. It might be supposed that the role of alanine in providing gluconeogenic substrate is diminished during prolonged fasting. It may also be possible that during prolonged fasting an equilibrium is established between muscle release and blood extraction for alanine.

With respect to blood sugar we found a relation between glucose and ketones after correction for age. This relation has been previously described in various studies of fasting experiments in children [6,9,10]. NEFA's are not related, and alanine demonstrates a weak correlation. Other studies showed a clear relationship between glucose and alanine [5,6,8,10]. The absence of a stronger relationship between glucose and alanine in our study can possibly be explained by a combination of factors, such as the relatively long period of fast (40 h), the age range (6-15 yr) and the adjustment for age with respect to this dependency.

With respect to ketones we found a negative relation between alanine and ketones. It has been suggested that ketones may serve as a protein sparing signal, diminishing muscle protein catabolism and alanine release. Ketone bodies should decrease alanine production by inhibition of proteolysis and branched chain amino acid catabolism [16,17]. The effect is a lower alanine level and a reduced gluconeogenesis in the presence of ketones. The negative relationship found in our study can support the existence of such a regulatory mechanism.

The observed alterations in blood substrates during fasting in children in our study have their significance in a complex of inter-related metabolic processes, like: glycogenolysis, proteolysis, gluconeogenesis, lipolysis and ketogenesis, to maintain the blood glucose level and to generate alternative substrates for an adequate body energy supply [10,13,18]. A number of fasting experiments in children have demonstrated diminished glucose homeostasis and increased ketogenesis in younger children compared to older ones [5,7,26]. Our results generally support this phenomenon in children owing to the finding of a clear interrelationship between glucose, ketones and age. Sex differences are found for ketones and alanine at 40-h fast. Age effects are evidently present for various blood components and the results indicate that younger children provide more ketones for energy supply during prolonged fasting than older ones. Parallel with this phenomenon, the glucose concentration and probably the utilization of this substrate are reduced in younger children.

By means of these reference values from the standardized fasting test we intend to screen young neurological patients with possible disturbances in energy metabolism.

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## **CHAPTER 3, section 2**

### **CEREBROSPINAL FLUID CONCENTRATION AND CEREBROSPINAL FLUID/ BLOOD RATIO OF FUEL RELATED COMPONENTS IN CHILDREN AFTER PROLONGED FASTING**

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## SUMMARY

In order to obtain information about blood and cerebrospinal fluid (CSF) concentrations, and CSF/blood ratio data of fuel related substrates at the end of a prolonged fast in children, we have selected biochemical data from fasting test procedures in 11 control children aged 3-5 yr, fasted 24 h, and 58 control children aged 6-15 yr, fasted 40 h. There was a good correlation between blood and CSF concentrations for glucose, acetoacetate and  $\beta$ -hydroxybutyrate. The relation with age and sex has been analyzed only in the older children. CSF and blood values for glucose are positively related with age, and both ketones are negatively related with age. Lactate, pyruvate and alanine concentrations in blood and CSF are not related with age, except for CSF pyruvate. With respect to the CSF/blood ratio for the above mentioned components, only the value for acetoacetate is sex and age related. The calculated median caloric values for the sum of glucose, lactate, pyruvate and ketones in CSF are independent of age at the end of a 40-h fast. The diminished glucose contribution on the CSF caloric homeostasis in younger children is fully compensated by the ketone bodies.

## INTRODUCTION

It is known that the brain can utilize ketones as an alternative energy supply. Generally, during (prolonged) fasting the contribution of glucose as the most important energy yielding substrate for brain diminishes. Simultaneously, there is an increase in acetoacetate and  $\beta$ -hydroxybutyrate utilization in brain.

Studies on fuel requirements of the central nervous system (CNS) under postabsorptive and fasting conditions have been performed by (1) catheterization techniques, which combine arteriovenous concentration differences with estimated brain blood flow [1-3], (2) radioisotopic and radioautographic techniques in brain [4,5], and (3) cerebrospinal fluid (CSF) and blood level studies of fuel related components [6,7]. As the CSF compartment reflects the (altered) CNS metabolism, data of CSF might give information about the metabolism of energy providing substrates in CNS.

Fasting experiments, especially in younger children, have demonstrated the tendency to develop hypoglycemia, and the rapid production and utilization of ketones to spare glucose [8-15]. It is known that disturbances in the brain energy provision in children can cause alterations in cerebral functions [16]. However, data in the literature about the metabolism of energetic substrates in CNS of children are very scarce. We have collected data of several fuel related components in blood and CSF in children after prolonged fasting. In this paper, data are presented of blood and CSF glucose, lactate, pyruvate, alanine,  $\beta$ -hydroxybutyrate and acetoacetate. The aim of this study was to obtain information about CSF concentrations of fuel related substrates, the CSF/blood ratios for these components, the CSF caloric homeostasis at the end of a prolonged fast in children, and the relation with age and sex. Furthermore, we intend to develop a model to detect disturbances in the energy balance of metabolism of the CNS in children.

## SUBJECTS AND METHODS

### Subjects

Over a period of five years a total of 69 children, 25 girls and 44 boys, ranging in age from 3-15 yr, were included in this study. These children were observed clinically because of intermittent behavioral problems and/or unexplained seizures, where the possibility of a hypoglycemia disorder was considered or had to be ruled out. They were classified retrospectively as suitable reference subjects because of lack of evidence for hypoglycemic and endocrine or metabolic abnormalities. Appropriate studies ruled out immunologic and chronic infectious diseases, deficiencies and disorders caused by toxic agents. Informed consent of the parents and of the older children themselves was obtained before the investigation.

### Fasting test procedures

The subjects were on a normal hospital diet for at least 72 h before the test. Fasting was always started after a last meal at 6 p.m. Only drinking of water was allowed during the fast. The length of fasting period was indicated by the child's age (table 1). For group I (age 3-5 yr), blood was sampled at the end of a 24-h fast, for group II (age 6-15 yr) at the end of a 40-h fast. Analysis of CSF components is a test which belongs to the standard laboratory examination of patients with possible disorders of CNS in our university department of child neurology. The lumbar puncture was always performed at the end of the fast in children.

Table I: Group characteristics of the subjects.

		Number		Age (mean $\pm$ SD)	
		Boys	Girls	Boys	Girls
Group I	3-5 yr	11	-	4.7 $\pm$ 1.0	
Group II	6-15 yr	33	25	10.2 $\pm$ 2.8	10.8 $\pm$ 2.8

### Biochemical analysis

Biochemical analyses of blood components have been reported previously [17].

These methods are also used for the analysis of the CSF components. For the assay of glucose, pyruvate and lactate in CSF, 1.0 ml CSF was directly mixed with 50 mg NaF and 50  $\mu$ l 1.75 mol/l citric acid, adjusted to pH 4.0 with NaOH (40%).

The caloric value of the fuel substrates can be calculated by assessing caloric values of 16.4 kJ/g glucose, 19.7 kJ/g  $\beta$ -hydroxybutyrate, 17.4 kJ/g acetoacetate, 15.2 kJ/g lactate and 13.0 kJ/g pyruvate [18].

### Statistical analysis

Statistical analysis was performed within the previously reported linear model [17]. However, due to the rather constant median level of the ketones in the children aged 6-10 yr, we fitted a two-phase regression model according to the technique described by Hinkley [19].

## RESULTS

### CSF components and CSF/blood ratios at the end of fast

In table II, we present the median concentrations of blood and CSF components, and the values of the ratio CSF/blood at the end of fast for group I (age 3-5 yr, 24-h fast) and group II (age 6-15 yr, 40-h fast). There was a good correlation between the blood and CSF concentrations for glucose ( $r = 0.55$ ), acetoacetate ( $r = 0.56$ ) and  $\beta$ -hydroxybutyrate ( $r = 0.71$ ). CSF lactate, pyruvate and alanine concentrations show no correlations with the blood concentrations. For glucose the CSF/blood ratio is always about 0.70. The CSF/blood ratios for lactate and pyruvate are about 1.0, and for alanine about 0.12. It is remarkable that the median CSF/blood ratio for acetoacetate is consistently twice that for  $\beta$ -hydroxybutyrate. The median CSF/blood ratios for acetoacetate and  $\beta$ -hydroxybutyrate are lower in group I than in group II. Because the number of children and the age range in group I are small, statistical analyses of the various biochemical parameters with respect to sex and age were not considered. In the older children group II, however, we have evaluated these matters.

Table II: Median blood and CSF values, and median ratios CSF/blood at the end of fast (respectively 24 and 40 h).

Parameter	Unit	Boys aged 3-5 yr (n=11); 24-h fast		
		Blood	CSF	Ratio
Glucose	mmol/l	3.5	2.5	0.69
Lactate	$\mu\text{mol/l}$	1720	1730	1.12
Pyruvate	$\mu\text{mol/l}$	166	155	0.90
Ratio L/P		10.8	11.9	
Alanine	$\mu\text{mol/l}$	291	47	0.15
$\beta$ -OH-butyrate	$\mu\text{mol/l}$	2200	250	0.10
Acetoacetate	$\mu\text{mol/l}$	560	130	0.20
Ratio $\beta$ -OH-B/AcAc		3.7	1.9	
Total ketone bodies	$\mu\text{mol/l}$	2656	380	0.13

Parameter	Unit	Boys and girls ages 6-15 yr (n=58); 40-h fast					
		Boys (n=33)			Girls (n=25)		
		Blood	CSF	Ratio	Blood	CSF	Ratio
Glucose	mmol/l	3.4	2.4	0.69	3.4	2.3	0.68
Lactate	$\mu\text{mol/l}$	1640	1680	1.12	1640	1680	0.98
Pyruvate	$\mu\text{mol/l}$	177	173	0.90	177	165	0.87
Ratio L/P		9.6	9.8		9.6	10.4	
Alanine	$\mu\text{mol/l}$	345	40	0.15	345	41	0.12
$\beta$ -OH-butyrate	$\mu\text{mol/l}$	3687	595	0.10	3687	346	0.14
Acetoacetate	$\mu\text{mol/l}$	723	266	0.20	723	174	0.27
Ratio $\beta$ -OH-B/AcAc		4.6	2.2		4.6	2.1	
Total ketone bodies	$\mu\text{mol/l}$	4444	870	0.13	4444	522	0.16

### Influence of sex and age on blood and CSF concentrations and CSF/blood ratios for the various components at the end of fast

Within the statistical model referred to, the effects of sex and age on the blood and CSF components, and on the ratio CSF/blood for group II after 40 h fast were tested for their significance. The results (p values of these test procedures) are presented in table III.

#### Sex

Blood and CSF values for glucose, lactate, pyruvate and alanine are not clearly sex related. Especially for children above 10 yr, there is evidence for a difference between boys and girls with regard to the blood and CSF concentrations of the ketone

bodies. For girls, the median total blood ketone concentrations are 20% lower and the median CSF concentrations are 40% lower than in boys (table II). Furthermore, with respect to the CSF/blood ratios only the ratio for acetoacetate is highly sex related.

### *Age*

Blood and CSF values for lactate, pyruvate and alanine are not clearly age related, except for CSF pyruvate. Age effects are indicated for the CSF as well as for the blood concentrations of glucose and individual (and total) ketones (table III). With respect to the CSF/blood ratios, only the ratio for acetoacetate is age related. In table IV, the mean value and the standard deviation are presented for the various CSF components in group II. Within the statistical model used, estimates  $p_{2.5}$ ,  $p_{50}$  and  $p_{97.5}$  are given for, respectively, the 2.5, 50 and 97.5 percentiles. For the variables that show clear age dependency, estimated percentiles are presented for different ages. The individual values of CSF/blood ratios, and the CSF and blood concentrations of glucose, acetoacetate and  $\beta$ -hydroxybutyrate together with the estimated percentiles (2.5 and 97.5 percentiles) for group II are presented in fig. 1. For comparison the values for group I are plotted in. For the interpretation of the figure it should be remembered that the age group 3-5 yr has fasted 24 h while older children have fasted during 40 h.

### **CSF caloric homeostasis**

In order to obtain information about the contribution of glucose, lactate, pyruvate and ketones to the CSF caloric value at the end of fast in relation to age and fasting time in children, we calculated this value for these components.

In our study, the median caloric value in CSF for the sum of glucose, lactate, pyruvate and ketones is independent of age for group II at the end of fast (10.8 kJ/ml CSF). Moreover, there is no difference between the median caloric value for group I and II (respectively 10.6 kJ/ml CSF and 10.8 kJ/ml CSF). The age course of the CSF caloric values is presented in fig. 2. In order to obtain information about the relative contribution of glucose to the total CSF caloric values in relation to age, we have given the ratio glucose joules/total joules in CSF in the same figure. The lower glucose

contribution in younger children is compensated by a higher ketone contribution.

Table III: p-values for statistical significance testing of sex and age effects on the blood and CSF concentrations and the ratios CSF/blood after 40-h fast for age group II (n=58).

Parameter	Test of sex effect			Test of age effect		
	Blood	CSF	Ratio	Blood	CSF	Ratio
Glucose	0.86	0.66	0.37	0.07	0.001 <sup>a</sup>	0.19
Lactate	0.64	0.58	0.51	0.09	0.42	0.12
Pyruvate	0.26	0.83	0.15	0.21	0.01 <sup>a</sup>	0.83
Ratio L/P	0.49	0.48		0.68	0.03 <sup>a</sup>	
Alanine	0.06	0.83	0.09	0.13	0.48	0.30
$\beta$ -OH-butyrate	0.06	0.03 <sup>a</sup>	0.66	0.0001 <sup>a</sup>	0.0001 <sup>a</sup>	0.90
Acetoacetate	0.29	0.005 <sup>a</sup>	0.002 <sup>a</sup>	0.001 <sup>a</sup>	0.0001 <sup>a</sup>	0.006 <sup>a</sup>
Ratio $\beta$ -OH-B/AcAc	0.02 <sup>a</sup>	0.44		0.0002 <sup>a</sup>	0.28	
Total ketone bodies	0.03 <sup>a</sup>	0.02 <sup>a</sup>	0.18	0.0001 <sup>a</sup>	0.001 <sup>a</sup>	0.58

<sup>a</sup> Statistically significant  $p < 0.05$ .

Table IV: CSF values after 40-h fast for 6-to-15-yr-old children.

CSF parameter	Unit	Mean	SD	Age	P2.5 <sup>a</sup>	P50 <sup>a</sup>	P97.5 <sup>a</sup>
Glucose	mmol/l	2.37	0.34	6	1.64	2.10	2.69
				12	1.90	2.43	3.11
				15	2.04	2.61	3.35
Lactate	$\mu$ mol/l	1694	201	6-15	1334	1682	2121
Pyruvate	$\mu$ mol/l	171	29	6	136	187	257
				12	118	162	223
				15	110	151	207
Ratio L/P		10.1	1.51	6	7.0	9.2	12.1
				12	7.8	10.3	13.5
				15	8.3	10.9	14.3
Alanine	$\mu$ mol/l	40	11	6-15	23	39	66
$\beta$ -OH-butyrate	$\mu$ mol/l	494	257	6-10	186	547	1610
				12	123	362	1064
				15	71	209	613
Acetoacetate	$\mu$ mol/l	225	104	6-10	96	252	664
				12	66	172	454
				15	39	104	274
Ratio $\beta$ -OH-B/AcAc		2.17	0.49	6-15	1.44	2.12	3.12
Total ketone bodies	$\mu$ mol/l	719	357	6-10	288	803	2235
				12	193	537	1494
				15	113	314	874

<sup>a</sup> Estimates within the lognormal model.

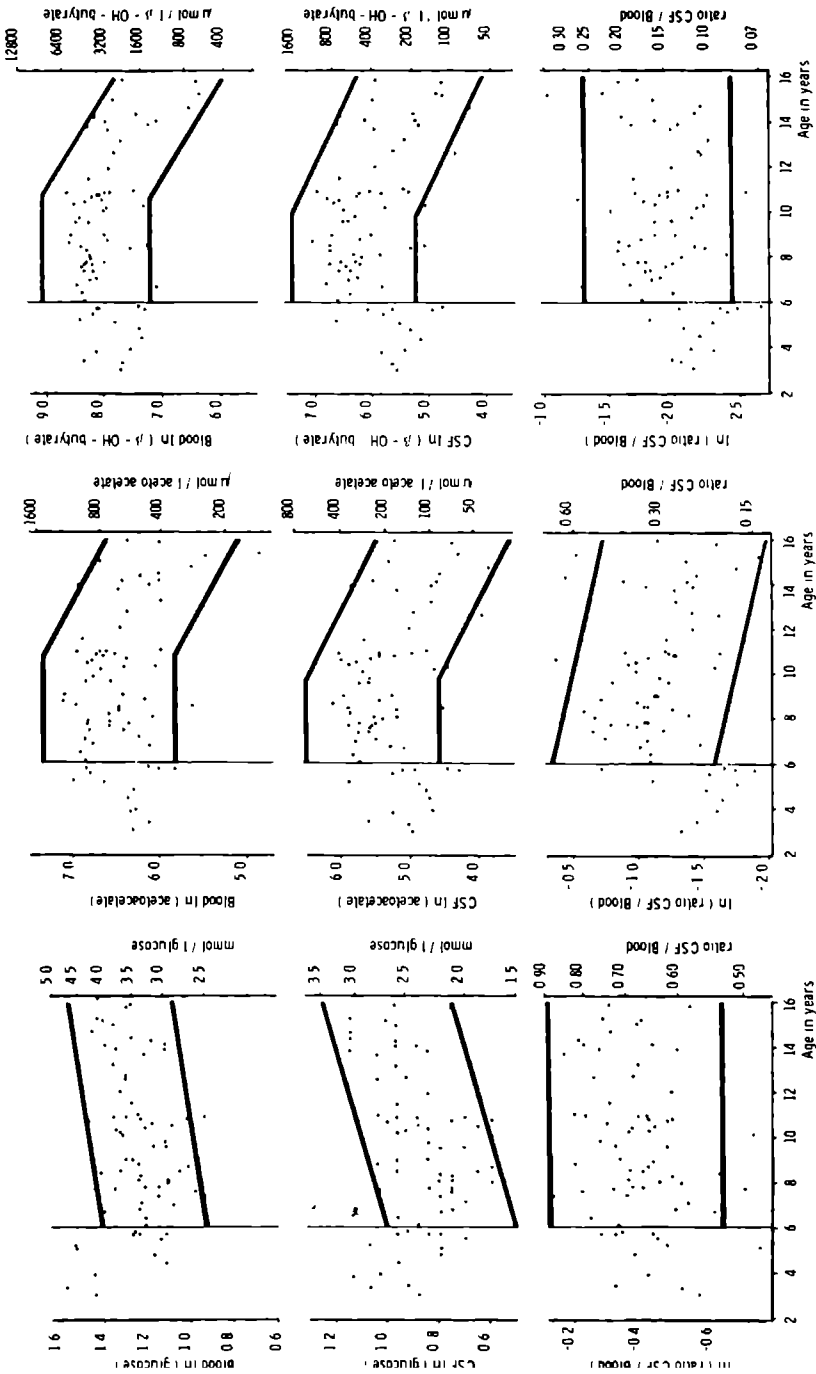


Figure 1: The estimated regression lines (2.5th and 97.5th percentiles) of glucose, acetoacetate and 3-hydroxybutyrate versus the age for blood and CSF ratios, and for the CSF/blood ratio are presented for group II. Individual values of group II are spotted in.



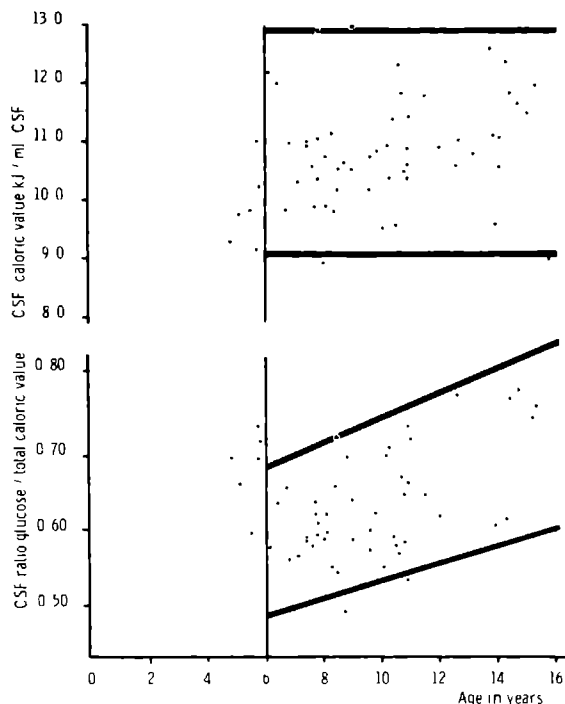


Figure 2: The estimated regression lines (2.5th and 97.5th percentiles) for both the total CSF caloric values and the caloric ratio glucose joules/total joules versus the age are presented for group II. Individual values of group I and group II are spotted in.

## DISCUSSION

Especially in children during fasting, ketone bodies have an important role in supplying fuel for brain energy requirements in order to spare glucose. Changes in blood concentration of fuel related substrates in children during fasting have regularly been observed ([17,20-23]. A number of fasting experiments in children have demonstrated that glucose homeostasis and ketogenesis are related to age [8,10,12-15,24]. The blood concentrations of fuel related substrates after a prolonged fast in children in this study agree well with other observations [8,10,12-14,25].

The transport of energy yielding components from blood to brain is regulated by the blood/brain barrier. Glucose enters the brain by a carrier mediated transport. A

common carrier mechanism is assumed for transport of lactate, pyruvate, acetoacetate and  $\beta$ -hydroxybutyrate [26]. Measurements of fuel related components in CSF might give information about the metabolism of energetic substrates in CNS, but we have to keep in mind that concentrations in CSF of above mentioned components are determined by the passage over the blood/brain barrier, the exchange with the brain extracellular fluid, and the bulk flow reabsorption by the arachnoid villi.

In our study, concentrations of glucose and ketone bodies in CSF show a clear correlation with the concomitant blood concentrations. Like the blood values, the CSF values for glucose and ketones show an age dependency at the end of fast. For ketones, we observed that children in the age range 6-10 yr have a distribution of blood and CSF values after a 40-h fast independent of age (fig. 1). This most likely represents a direct effect of ketone bodies both on the adipose tissue (antilipolytic effect) [27,28] and on the rate of ketogenesis by means of inhibiting free fatty acid oxidation [29,30]. This feedback mechanism might play an important role in preventing the development of uncontrolled hyperketonemia during starvation. Furthermore, an even more pronounced sex dependency is found for ketones in CSF than in blood. Maybe, a hormonal regulation is responsible for this phenomenon, as the difference is especially present in the oldest children. Also an effect of different weight or body mass can be an explanation for this phenomenon.

With respect to the CSF/blood ratios for the various components, the ratio for glucose is relatively stable, independent of the blood concentration or age. The value agrees well with other observations [7]. The ratio for acetoacetate is consistently twice that for  $\beta$ -hydroxybutyrate. In CSF/blood studies in adult persons, Owen et al [7] found the same result at the end of a prolonged fast. In arteriovenous extraction studies in brain, Persson et al [1] also observed that the uptake of acetoacetate by brain of infants and children is twice that for  $\beta$ -hydroxybutyrate at any given blood concentration. Studies in dogs during fasting ketonemia and also after blood infusions of ketones demonstrated the same preferential brain uptake of acetoacetate both by measurements of CSF/blood ratios as by arteriovenous extraction methods [6].

With respect to age effects, a clear negative relation was only observed between age and CSF/blood ratio for acetoacetate. Surprisingly, the ratio for  $\beta$ -hydroxybutyrate

did not show any age dependency. In rats, Cremer et al [31] observed a decrease of brain uptake capacity for  $\beta$ -hydroxybutyrate with age. Settergren et al [3] found also higher brain uptake rates for ketones in children compared to adults.

Summarizing for the ketones, the following provisions during fasting in children can be concluded from our results: (1) the increase during fast of blood and CSF ketones is age related, (2) an age and sex relation for the CSF/blood ratio of acetoacetate.

One might tentatively postulate that CSF/blood ratio data give information about transport capacity for ketones from blood to CSF/brain. In a study of ketones transport from blood to brain in dogs after prolonged fast, Wiener et al [6] found comparable results both with CSF/blood ratio studies as with arteriovenous extraction studies. Therefore, the age related changes of CSF/blood ratio for ketones might be interpreted as a facilitation of the transport of blood ketones to brain in younger children.

The median CSF caloric values in children at the end of a 40-h fast are independent of age. A lower glucose level in CSF in younger children is fully compensated by a higher ketone level, suggesting that ketone bodies contribute to CSF caloric homeostasis and compensate for diminished glucose provision. Furthermore, it is striking that the median values of the CSF caloric values in children of group I, fasted 24 h, and of group II, fasted 40 h, are almost equal. The concentration of these substances in CSF may in some way reflect the availability of these fuel substrates to the CNS for oxidation.

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## **CHAPTER 3, section 3**

### **FUEL-RELATED COMPONENTS IN CSF AND BLOOD AFTER PROLONGED FASTING IN CHILDREN WITH ENCEPHALOPATHIES OF UNKNOWN ORIGIN**

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**SUBMITTED FOR PUBLICATION**

## SUMMARY

Alterations in the cerebral energy supply are likely to cause cerebral function disturbances. Fasting is a suitable method for studying the energy metabolism. As the cerebrospinal fluid (CSF) compartment reflects the brain metabolism, data in CSF might give information about the metabolism of fuel substrates in brain. Children aged 6-15 yr with encephalopathies of unknown origin were fasted for 40 hours. We compared the biochemical data on several fuel-related components in blood and CSF at the end of the fast with the values of a reference group of children. In patients with mental retardation and behaviour disorders only the blood glucose level was significantly higher than that in the reference group. In patients with complex partial epilepsy many significant abnormalities were found, especially low CSF ketones. In patients with primary generalized epilepsy no abnormalities were found. The possible significance of the observed abnormalities in concerned patients are discussed.

## INTRODUCTION

The brain (especially the cerebellum) has a very low energy reserve and one of the highest metabolic rates in the body. To meet its energy requirements, glucose as well as ketones form a major source of fuel for the brain. Ketones are especially important in young children during fasting because they not only provide fuel, but also help to conserve glucose (Owen et al. 1974, Settergren et al. 1975, Saudubray et al. 1981, Lamers et al. 1987). Cerebral function is closely linked to energy metabolism: cerebral glucose utilization directly reflects the brain's level of functional activity (Sokoloff et al. 1977). Fasting is a suitable method for studying (disturbances in) the energy metabolism of the brain (Saudubray et al. 1981). Alterations in the cerebral energy supply are likely to cause cerebral function disturbances.

In this study, children with encephalopathies of unknown origin were investigated to evaluate whether there was any underlying pathophysiology of the cerebral energy metabolism. This group comprised children with mental retardation and behaviour disorders and children with cryptogenic or non-lesional epilepsy. Furthermore, also children with perinatal problems or with congenital malformations of the central nervous system (CNS) and paroxysmal behaviour disturbances not caused by pedagogic or psychosocial factors were included in this study. We collected biochemical data on several fuel-related components in the children's blood and CSF after prolonged fasting procedures and compared the results to those obtained from a reference group of children.

## SUBJECTS AND METHODS

### Subjects

Over a period of five years, we collected data on a total of 176 children. All the children had undergone appropriate tests to rule out endocrinological or metabolic abnormalities, immunological and chronic infectious diseases, deficiencies and disorders caused by toxic agents, chromosomal abnormalities and syndromes. Informed consent was obtained from the parents and the older children themselves



before enrolment in the study. The population also included a group of 58 children who served as suitable non-organic reference subjects. These reference children were referred to our clinic because of intermittent behaviour problems, headaches and/or unexplained seizures, with the aim of detecting or ruling out a biochemical, especially hypoglycaemic, disorder.

The study subjects were divided into 5 groups: group 1 had perinatal problems and paroxysmal behaviour disturbances (n=21), group 2 had congenital malformations of the CNS and paroxysmal behaviour disturbances (n=30), group 3 had mental retardation of unknown etiology without epilepsy (n=46), groups 4 and 5 had cryptogenic or non-lesional epilepsy; group 4 comprised patients with primary generalized epilepsy (n=10) and group 5 patients with complex partial epilepsy (n=11). Data on the epilepsy patients were collected in a seizure-free period. The antiepileptic drugs used by the epilepsy patients are presented in Table 1. The administration of these drugs was not changed in any way during the testing period.

Table 1. Medication regime for epilepsy groups 4 and 5.

medication	group 4 (n=10)	group 5 (n=11)
valproate	1	1
valproate + phenobarbital	1	0
phenobarbital	1	1
phenobarbital + diphenoin	0	2
phenobarbital + carbamazepine	0	3
carbamazepine	4	2
no medication	3	2

### Fasting test procedure

All the subjects had been eating normal hospital meals for at least 72 h before testing. In each case, fasting was started after the last meal of the day at 6 p.m. The children (age 6-15 yrs) were fasted for 40 hours. At the end of the fasting period, blood samples were taken and CSF was obtained via a lumbar puncture (at our University Department of Child Neurology, the analysis of CSF components is a standard laboratory examination for patients with possible disorders of the CNS). The standard fasting procedure has been described previously (Lamers et al. 1987).

## Biochemical analysis

In a previous report on the patients who served as the reference group, we described the biochemical methods and presented the concentrations of blood and CSF components, and the calculated CSF caloric values (Lamers et al. 1987). The following parameters were tested in CSF: glucose, alanine, total ketones (=  $\beta$ -hydroxybutyrate + acetoacetate), lactate and the CSF caloric value; in blood: glucose, alanine, total ketones (=  $\beta$ -hydroxybutyrate + acetoacetate), lactate and non-esterified fatty acids (NEFAs). To measure the CSF caloric value, we calculated the sum of the caloric values for glucose, lactate, pyruvate and ketones. The ratio CSF:blood for glucose, alanine and ketones was also calculated.

## Statistical analysis

Statistical analyses were performed within the previously reported linear model (Lamers et al. 1985). A one-sample student t test was used to test the significance of age-corrected patient residuals. Age-corrected patient residual:

$$R_{\text{pat}} = \frac{X_{\text{pat}} - \bar{X}_{\text{ref}}}{S_{\text{ref}}}$$

( $X_{\text{pat}}$  = log. concentration in patient;  $\bar{X}_{\text{ref}}$  = mean of the log. concentration in reference children of the same age as the patient;  $S_{\text{ref}}$  = standard deviation of the log. concentration in reference children of the same age as the patient).

We calculated a 'median percentage' (MP) for each separate parameter in each patient group, using the formula:

$$\frac{\text{observed patient value}}{\text{median reference value at patient age}} \times 100\%$$

## RESULTS

The MP values and the results of the significance tests are presented in Table 2. There were no significant differences between the reference group and the patients with perinatal problems or the patients with congenital malformations of the CNS, with the exception of a lower CSF alanine in the congenital malformation group ( $p = 0.04$ ,  $MP = 86$ ). The blood glucose level in the group of patients with mental

retardation was significantly higher than that in the reference group ( $p = 0.01$ , MP = 107) and the CSF glucose level showed the same tendency ( $p = 0.06$ , MP = 105). There were no significant differences between the primary generalized epilepsy group and the reference group, whereas for the complex partial epilepsy group, remarkable differences were observed: the blood lactate and alanine levels were significantly lower ( $p < 0.01$ , MP = 70 and 83, respectively), the CSF lactate concentration ( $p = 0.03$ , MP = 91), the CSF ketone concentration and the CSF:blood ratio for ketones were also significantly lower ( $p < 0.01$ , MP = 64 and 80, respectively); the blood ketone levels (both acetoacetate and  $\beta$ -hydroxybutyrate) were lowest in this group but the differences were not significant ( $p = 0.07$ , MP = 81). There was no significant difference in the CSF caloric value between the reference group and any of the 5 groups of study subjects.

Table 2. Median percentage\*\*\* (MP) of blood and CSF parameters in the 5 patient groups

	Group 1 perinatal problems n=21	Group 2 congenital malformations of the CNS n=30	Group 3 mental retarda- tion without epilepsy n=46	Group 4 epilepsy primary generalized n=10	Group 5 epilepsy complex partial n=11
Blood					
glucose	101	105	107**	98	101
lactate	106	97	108	97	70**
alanine	101	94	98	89	83**
ketones	94	90	92	93	81
NEFAs	100	108	111	112	112
CSF					
glucose	101	102	105	99	103
lactate	105	103	104	99	91*
alanine	108	86*	102	89	89
ketones	92	92	86	81	64**
caloric value	102	100	103	96	97
CSF:blood ratio					
glucose	100	97	98	100	104
alanine	100	95	108	95	115
ketones	90	102	97	91	80**

\*  $0.01 < P < 0.05$

\*\*  $P \leq 0.01$

\*\*\*  $MP = \frac{\text{observed patient value}}{\text{median reference value at patient age}} \times 100\%$

## DISCUSSION

No significant abnormalities were found in the children with perinatal problems and the children with primary generalized epilepsy. We were unable to find an explanation for the isolated observation of a reduced CSF alanine level in the group of patients with congenital malformations of the CNS and there were no indications in the literature for alanine involvement in such cases. In the patients with mental retardation only the blood glucose level was significantly higher. Various factors may have been responsible for this: 1) inadequate fasting, 2) higher gluconeogenesis, 3) lower blood-brain glucose transport or 4) lower glucose utilization by a retarded brain. We can exclude inadequate fasting because the children were under constant observation during the fasting period and their blood NEFAs and ketone levels indicated normal lipolysis and ketogenesis during fasting. Increased gluconeogenesis is conceivable, but the blood levels of the glyconeogenic substrates: alanine (from muscle proteolysis) and lactate (from anaerobic glycolysis) were normal, which indicates the normal provision of substrates for gluconeogenesis. Lower blood-brain glucose transport can also be excluded on the grounds of reports in the literature and our results. Lund-Andersen et al. (1977) found that the transport of glucose from the blood to the metabolic apparatus of the neuron and glia cells of the brain involves 3 steps: 1) transport across the blood-brain barrier (BBB), 2) diffusion to the neuron and glia cell membranes and 3) transport across the nerve and glia cell membranes. Recently, persistently low CSF glucose levels and low CSF:blood glucose ratios (0.19 to 0.35) were described by De Vivo et al. (1991) in two young children with seizures and developmental delay. These authors demonstrated a partial defect in the type I glucose transporter in erythrocytes. This erythrocyte transporter protein appears to be identical to the glucose transporter type I present in brain capillaries. Such a defect can be expected to interfere with cerebral energy metabolism and brain function. In our study, the CSF:blood glucose ratio in each individual mentally retarded patient was normal, which therefore excludes a glucose transport defect in their BBB. Lower cerebral glucose utilization cannot be excluded. Fiorelli et al. (1992) reported lower cerebral glucose utilization in patients with myotonic dystrophy (MD). Using PET

scanning, these authors demonstrated a reduction in the  $^{18}\text{F}$ -labelled 2-fluoro-2-deoxy-D-glucose (FDG) rate constant  $K_1$  of the blood-to-brain glucose transporter in MD patients. A possible defect in the function of the insulin receptor on the BBB in MD patients has been considered (Moxley et al. 1984). Fiorelli et al. (1992) concluded that the lowered glucose utilization in MD brains could not be explained by a glucose transporter defect, but it could be attributed instead to a primary reduction in brain metabolic needs. Jagust et al. (1991) observed similar glucose transport reduction (20%) using PET scanning on adults with Alzheimer's disease. The decline in glucose transport in Alzheimer's disease is more likely related to the cortical atrophy and to the decline of functional activity of the surviving tissue (Kalaria et al, (1989) and Jagust et al, (1991)). It has been established that the glucose transport across the neuron membranes far exceeds the rate of glucose metabolism in neurons. Therefore, in our patients with mental retardation, a lower glucose metabolism/utilization in the brain itself could have been responsible for the higher blood and CSF glucose levels. We could not find any data about altered glucose metabolism, including PET scan disturbances, in the literature on pure mentally retarded children. However, it is known that patients with mental retardation show a slow background activity for their age on an EEG, which indicates reduced brain functioning. This phenomenon may also indicate that the brain has lower metabolic needs.

Many abnormalities were observed in the patients with complex partial epilepsy. The lower blood alanine level in combination with lower blood lactate may have been caused by reduced proteolysis and alanine delivery from muscle tissue during fasting or an increased gluconeogenesis. The normal blood glucose level does not indicate an alteration in gluconeogenesis. The CSF ketone levels and the CSF:blood ketone ratios were also significantly decreased (MP = 64 and 80, respectively). The anticonvulsant medication may have been responsible for the above-mentioned abnormalities in these patients, because it is known that antiepileptic drugs cause liver enzyme induction (Perucca et al. 1984). In 7 epileptic children, Holowach Thurston et al. (1983) demonstrated that chronic valproate administration (mean daily dose of 38 mg/kg) reduced the level of ketonaemia after an overnight fast, probably by inhibiting fatty acid oxidation. Although we could not find any clear differences in the medication

type and daily dose between our group 4 with primary generalized epilepsy and group 5 with complex partial epilepsy, it was striking that the biochemical abnormalities, especially the lower CSF ketone levels, were only observed in the complex partial epilepsy patients. The low CSF ketone levels in our study can partly be explained by the concomitant low blood levels. A substantial proportion of the lower CSF ketones can be explained by 1) defective ketone transport over the BBB (the MP of CSF:blood ketones ratio was 80) and/or 2) by increased brain ketones utilization. Gjedde et al. (1975) reported that blood ketone extraction by the brain in contrast to blood glucose extraction, increased significantly with an increasing duration of starvation, which indicated that the transport mechanism adapted to ketonaemia. Moreover, the cerebral extraction of ketones is known to be much higher in young infants than in older children and adults (Kraus et al. 1974, Settergren et al. 1976). However, in our study all the children were fastened for the same period (40 hours) and all the significance tests were performed after correction for age effects, so we can exclude alterations caused by differences in the fasting duration or age. In our study, we could not distinguish ketone transport (adaptation) disturbances from increased brain ketones utilization in our low CSF:blood ketone ratio. The altered energy metabolism in our complex partial epileptic patients may have been caused by alterations in endocrine function, because the conversion and provision of fuel substrates are also regulated by hormones. It is known that the serum prolactin level always increases after a seizure involving the temporal lobes in complex partial epilepsy (Pritchard et al. 1983). One possible explanation is that the limbic structures serve as a trigger for subcortical discharges on the hypothalamus, which regulates anterior pituitary secretions (Sperling et al. 1986). However, changes in the serum level of other endocrine functions, such as growth hormone, thyroid-stimulating hormone and cortisol, were not observed in complex partial epilepsy patients after a seizure or in the seizure-free period (Pritchard et al. 1983). In the light of these observations, we can exclude the possibility of neuroendocrine disturbances as a cause for the altered fuel metabolism in our patients. It has been suggested that the anticonvulsant effect of ketones on patients with epilepsy, which can be generated by fasting or a ketogenic diet, is due to metabolic adaptation of the brain metabolism to

ketones utilization (Appleton et al. 1972, Uhlemann et al. 1972, De Vivo et al. 1978). When Huttenlocker (1976) prescribed two ketogenic diets in an experiment on childhood epilepsy, he found support for a direct anticonvulsant action of ketones. Similarly, in two young children with a glucose transporter type I defect, who suffered from seizures (De Vivo et al. 1991), treatment with a ketogenic diet resulted in nearly normal subsequent neurological development as well as cessation of the seizures. These observations stress the possible relevance of brain ketones utilization in epilepsy and the significance of our finding of a low CSF ketone level during fasting in complex partial epilepsy.

In conclusion, in our patients with mental retardation with an unknown etiology and without epilepsy, a lower brain glucose demand (utilization) could have been responsible for the higher blood and CSF glucose levels. Further investigations with PET scanning might elucidate this phenomenon. In the patients with complex partial epilepsy, an alteration in brain ketone transport and/or utilization could have been responsible for the lower CSF ketone levels. As ketogenic diets have seldom been prescribed for patients with complex partial epilepsy, it would be interesting to study the effect of ketogenic diet treatment on these patients.

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## **CHAPTER 4, section 1**

### **AGE-RELATED CHANGES OF NEURON-SPECIFIC ENOLASE, S-100 PROTEIN AND MYELIN BASIC PROTEIN CONCENTRATIONS IN CEREBROSPINAL FLUID**

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## SUMMARY

Studies on cerebrospinal fluid (CSF) concentrations of neuron-specific enolase (NSE), S-100 protein (S-100) and myelin basic protein (MBP) in patients with neurological lesions indicate a quantitative relation between the degree of cell damage in the central nervous system (CNS) and the concentration of these CNS-specific proteins in CSF. Thus NSE, S-100 and MBP could be of use as markers for destructive processes in the CNS. We collected 937 specimens of CSF from children and adults ranging in age from newborn to 91 years, who were undergoing a diagnostic lumbar puncture for several clinical indications. Of these, 79 samples from subjects ranging in age from 0.7 to 66 years could be used retrospectively to construct a reference interval according to our criteria. In these 79 reference samples no sex-dependency existed. The relative increase of NSE, S-100 and MBP with age was similar (1 % per year), suggesting a common underlying mechanism. These results emphasize the necessity of using age-matched reference values when the CNS-specific proteins are to be evaluated in neurological diseases. We also present three case histories to discuss the possible clinical relevance of the measurement of NSE, S-100 and MBP in children and adults.

## INTRODUCTION

The clinician dealing with neurological disorders has to answer three questions. Is there a disease involving the nervous system? If so, where is the disease located? What kind of disease is it, what is its pathological nature? The first question is often the most difficult one to answer. To answer it, but also the third question, the assessment of damage to the central nervous system (CNS) by determination of neuron-specific enolase (NSE), S-100 protein (S-100) and myelin basic protein (MBP) in cerebrospinal fluid (CSF), may be helpful. NSE, S-100 and MBP are regarded as nervous-system-specific proteins. NSE, the  $\gamma\gamma$ -isoenzyme of enolase, is a soluble cytoplasmic protein localized mainly in neurons (1). S-100, named after its solubility in 100% saturated ammonium sulfate at neutral pH, constitutes a major component of the cytosol predominantly of glial cells (2,3). MBP is detectable in developing oligodendroglia and is bound to cellular membranes of central and to a lesser extent peripheral myelin (4,5). Thus, increased concentrations of NSE, S-100 and MBP in CSF indicate CNS damage and may help to identify the cell type or part (neuron, glia or myelin) affected by the pathological process. NSE, S-100 and MBP can now be measured with sufficient sensitivity by immunoassays (1-4). However, data on sex- and age-related reference values are lacking.

We measured the concentrations of NSE, S-100 and MBP in CSF from 937 children and adults, ranging in age from newborn to 91 years, who were undergoing a diagnostic lumbar puncture for several clinical indications. To establish possible sex- and age-related effects in the concentrations of NSE, S-100 and MBP, we retrospectively selected, according to certain criteria, 79 of the patients as a reference group.

We also present three case histories to discuss the possible clinical relevance of the measurement of NSE, S-100 and MBP in CSF.

## MATERIALS AND METHODS

### Reference group

From 1985 to 1988, 937 CSF samples were obtained from patients undergoing

a diagnostic lumbar puncture for conventional clinical indications such as suspected CNS infection or neurological disorder. Of each CSF sample 0.5 mL was used for the present investigation. Retrospectively, we were able to use 79 of the samples (from 37 females and 42 males; age 0.7 - 66 years) for the formation of reference ranges. These samples were selected according to the following criteria: no use of medication, no evidence of an organic neurological disorder, an inherited metabolic disease, or a malignant disease; and normal concentration of total protein in CSF. The diagnoses selected in this way, comprise predominantly somatoform disorders [6]. To get normal controls subjects in a hospital population remains a problem: although we started with nearly 1000 cases, only 79 could be used for the formation of reference ranges. Relaxing our entry criteria led to qualitative differences in the reference ranges; e.g., when we included patients with migraine, the overall reference ranges significantly increased. So we used strict entry criteria.

### **Determinations in CSF**

NSE in serum and CSF were determined by radio-immuno assay (RIA) according to instructions of assay manufacturer (Pharmacia Diagnostics AB, Uppsala Sweden). This double antibody assay contains human NSE as antigen and rabbit anti-human antiserum. Sepharose-anti-rabbit IgG, raised in sheep is used as precipitating reagent. The detection limit for NSE is 2  $\mu\text{g/L}$ .

S-100 concentrations in CSF were determined by a particle-counting-immunoassay (PACIA), essentially as described by Sindic et al. (3). The S-100 immunoassay kits were received from C.J.M. Sindic of the department of Experimental Medicine, University of Brussels. The detection limit is 0.5  $\mu\text{g/L}$ .

MBP was determined by a double-antibody RIA kit according to the instructions of the manufacturer (catalog no DSL 1500; Diagnostic Systems Laboratories, Webster TX). Human MBP (whole molecule) is used as the antigen and rabbit anti-human MBP as antiserum. Goat anti-rabbit gamma globulin is the MBP precipitation reagent. The detection limit is 0.2  $\mu\text{g/L}$ .

## Statistics

After logarithmic transformation, we performed a regression analysis to yield age-related reference intervals for NSE, S-100 and MBP. We estimated the median value and the reference limits as the 5<sup>th</sup> and 95<sup>th</sup> percentiles. P-values for sex- and age-dependency were calculated.

The scatter diagrams of NSE and MBP against age contained no clear outliers. The scatter diagram of S-100 against age contained one clear outlier (7 µg/L at age 7 years), which we removed before calculating the reference intervals for S-100.

## RESULTS

### Age- and sex-dependency

NSE, S-100 and MBP in CSF increase with age. Table 1 lists the age-related reference intervals (µg/L). Figure 1 illustrates the NSE, S-100 and MBP concentrations as functions of age (years). The P-values for age-dependency were 0.03 for NSE, < 0.001 for S-100 and 0.001 for MBP. The 95% confidence intervals for the relative increase of NSE, S-100 and MBP with age overlapped and were as follows (percentage increase per year): NSE 0.1-1.4%; S-100 0.4-1.5%; MBP 0.5-1.9%.

There were no significant differences between males (n = 42) and females (n = 37) in age or in NSE, S-100 and MBP concentrations (0.20 < P < 0.80). Therefore we combined the data for further analysis.

Table I. Reference values (µg/L) for NSE, S-100 and MBP in CSF.

age in years	NSE			S-100			MBP		
	P5 <sup>a</sup>	P50	P95	P5	P50	P95	P5	P50	P95
1	2.2	5.0	10.2	0.9	1.5	2.6	0.12	0.30	0.72
20	2.7	5.8	12.0	1.1	1.8	3.3	0.17	0.40	0.95
40	3.1	6.5	13.8	1.3	2.2	4.0	0.22	0.52	1.21
60	3.8	7.8	16.0	1.6	2.7	5.0	0.30	0.70	1.57

<sup>a</sup>P = percentile

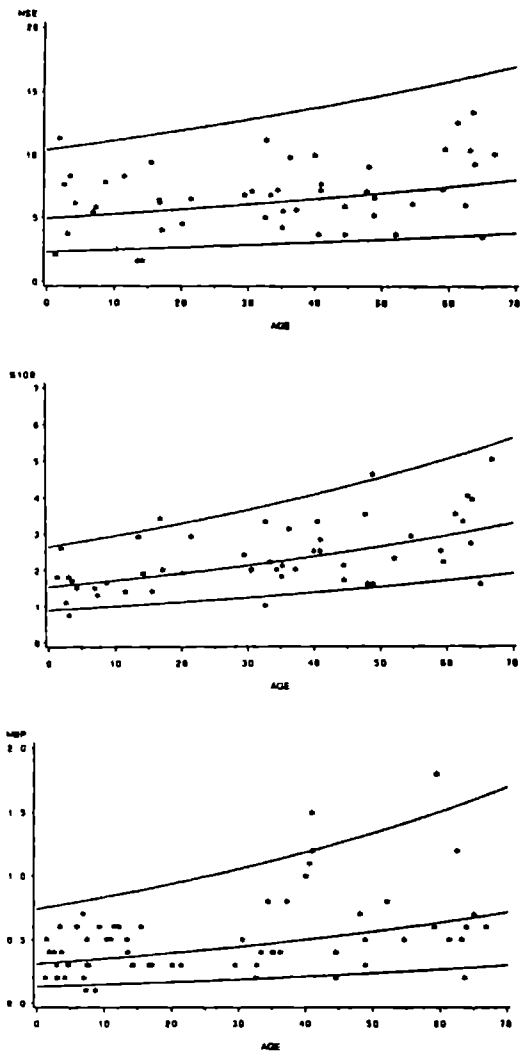


Figure 1: Scatter diagram of CSF concentrations ( $\mu\text{g/L}$ ) as a function of age (years) for reference sample patients: (top) NSE, (middle) S-100, (bottom) MBP. Solid lines indicate 5-95% confidence limits and median values.

### Clinical examples

Serial analysis of NSE, S-100 and MBP in CSF has been recommended in the literature as a very sensitive but unspecific screening variable for pathological organic CNS processes (2,4,7-9). In our opinion, these measurements have even greater

clinical relevance and potentials. To illustrate this we present three case histories (table II).

Table II: Details of clinical case histories.

Patient and diagnosis	Age in years	Duration of sickness in days	Concentration in $\mu\text{g/l}$		
			NSE (2.2-10.2) <sup>a</sup>	S-100 (0.9-2.6)	MBP (0.12-0.72)
<i>Acute transverse myelopathy</i>					
1 <i>Borrelia myelopathy</i>	6	5	7.3	2.8	20.0
		Treatment started			
		13	7.2	3.1	10.9
		27	9.7	2.1	0.4
2 Adenovirus myelopathy (untreated)	10	11	3.9	2.2	12.9
		23	7.3	2.5	1.1
<i>Acute panencephalitis</i>					
3 Herpes encephalitis	1.5	10	44.8	8.6	8.7
		Treatment started			
		24	11.0	1.9	2.9
		40	8.1	1.3	4.2
		49	5.9	1.5	-

<sup>a</sup> Reference interval (5<sup>th</sup>-95<sup>th</sup> percentiles) given in parentheses.

### *Transverse myelopathy.*

Patients with an acute transverse myelopathy show distinct nervous-system-specific protein profiles in CSF, depending on the different underlying causes and corresponding with different prognosis. Patient 1, with acute transverse myelopathy after infection with *Borrelia Burgdorferi* treated with penicillin G and patient 2, affected after adenovirus infection, show MBP values solely above the reference interval. The values of NSE and S-100 in the acute stage of the disease are within the respective reference intervals. This combination indicates acute and isolated demyelination. If this process terminates spontaneously (patient 2) or after treatment (patient 1), remyelination and clinical recovery occur. No neurological deficit remains because there was no irreversible damage to the neuron compartment.

In these cases, the determination of NSE, S-100 and MBP may serve to assess which compartment (neuron, glia or myelin) is affected. By elucidating which functional structure is involved in an acute neurological disorder, the CNS-specific



proteins can serve as a diagnostic but also as a prognostic tool.

### *Panencephalitis.*

In the CSF of a child (case 3) with an acute disease with fever, focal epilepsy and hemiparesis, we found clearly increased values for NSE, S-100 and MBP. Such an acute and severe panencephalitis has to be treated as a herpes encephalitis. The remarkably high values of NSE correspond to a very bad prognosis when therapy is delayed. After therapy was started, NSE and S-100 values decreased in our patient (table II). Thirty days after the start of the therapy, MBP was still clearly above the reference interval, indicating ongoing demyelination.

In this case the CNS-specific proteins may again serve as a diagnostic and prognostic tool. Another possible use is for evaluation and measurement of the effect of therapeutic interventions.

## DISCUSSION

NSE, S-100 and MBP in these CSF samples increased with age from 0.7 until 66 years. It is remarkable that the relative increases of NSE, S-100 and MBP with age are similar. The 95% confidence limits of the relative increases of NSE, S-100 and MBP overlap. The median increase of the CNS-specific proteins is 1% per year.

We found no prior reports on age dependency of CNS-specific proteins in the literature.

A supposed age-dependent increase in blood-brain barrier permeability, resulting in an increase in CSF total protein with age, cannot be the explanation for the age-dependency of the CSF CNS-specific proteins. Because they originate from the nervous system, there is no transudation of these proteins from the serum to the CSF across the blood-brain barrier. At least three explanations for increases in NSE, S-100 and MBP with age are possible: a) the age-dependency reflects increasing cell and myelin loss with age, b) the NSE, S-100 and MBP concentrations in the cells and myelin increases with age, whereas the turnover of the cells and myelin remains constant or, c) the same relative increase of NSE, S-100 and MBP (1% per year) with

age, could be the result of a common underlying mechanism, e.g., an increased half-life attributable to a reduced CSF bulk flow at older ages. The CSF bulk flow reportedly decreases with age (10), resulting in a synchronous increase of the concentrations of proteins (10-12).

Some investigators have reported about NSE, S-100 and MBP in CSF of children and adults in relation to various neurological diseases: head injury (2,9); CNS tumors (4,7,8,13-15); cerebral ischemia, including stroke (2,7,8,14-16); inflammatory diseases of the CNS, such as encephalitis or myelitis (3,7,13,14); multiple sclerosis (3,7,8); Guillain-Barre syndrome (3,7); epilepsies (8,14); migraine (8); cervical myelopathy (7,8); amyotrophic laterosclerosis (7); Huntington disease (17); Creutzfeldt-Jacob disease (18); dementia (8) and subarachnoidal and intracerebral bleeding or hematoma (2,7). We shall discuss the clinical potentials and restrictions of the CNS-specific proteins in relation to neurological diseases more in general.

Many modern imaging techniques yield important information concerning the status of the brain or spinal cord but cannot distinguish between irreversibly damaged brain or spinal cord (infarcts) on the one hand and reversible changes in viable tissue (edema) on the other. Although the neurological deficit depends on the size as well as on the site of the lesion -a small strategically placed lesion can lead to substantial clinical deficit- quantification of CNS-specific proteins may contribute to the more-detailed estimation of the actual structural and irreversible brain or spinal cord damage in various clinical situations (19). Therefore assessment of CNS-specific proteins can be of diagnostic and prognostic value (2,7-9). Markers of CNS cell damage can also be of clinical value in evaluating the effect of therapeutic measures to reduce cerebral cell damage caused by vascular reconstruction, hemodilution, hyperventilation, and treatment with barbiturates, calcium blockers, or mannitol (2). Although the nervous-system-specific proteins are very sensitive indexes (13), normal NSE, S-100 and MBP do not exclude disease. The time of CSF sampling in relation to the ictus of the cytolytic process is of crucial importance: in ischemic stroke patients, concentrations of NSE and S-100 were increased only between 18 h and 4 days after the ictus (2). In demyelinating diseases, MBP is cleared within 5 - 7 days after the ictus (5). Therefore, serial measurements can elucidate the dynamics of the patholo-

gical process in relation to therapy.

From our own clinical examples and review of the literature, we think the significance of the assessment of NSE, S-100 and MBP in clinical (child)neurology could be:

- to help to determine if the disease is involving the nervous system;
- to differentiate between damage to the glia or neuron compartment (clinical symptoms and signs, neuroradiology and neurophysiology also contribute to this differentiation, but in a less direct and specific way);
- to differentiate between irreversible damage or reversible changes in nervous tissue (in addition to a diagnostic value, there is a potentially prognostic one) and
- to help in evaluating the effect of treatment.

In this study we obtained reference values for NSE, S-100 and MBP in children and adults of different ages and both sexes. The availability of these age-matched reference values allows interpretation of CNS-specific proteins in CSF of patients with different neurological disorders, perhaps leading to a better understanding of their basic mechanisms.

In a second paper we intend to use age (but not sex) matching to investigate whether the CNS-specific proteins behave independently and show distinct profiles in various neurological disorders.

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## **CHAPTER 4, section 2**

### **NEURON-SPECIFIC ENOLASE, S-100 AND MYELIN BASIC PROTEIN IN THE CSF OF CHILDREN AND ADULTS WITH NEUROLOGICAL DISORDERS**

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RA Wevers**

**SUBMITTED FOR PUBLICATION**

## SUMMARY

In this study levels of neuron-specific enolase (NSE), S-100 protein (S-100) and myelin basic protein (MBP) in cerebrospinal fluid (CSF) of children and adults with distinct neurological disorders were examined. The median concentration level of the 3 proteins between 19 different neurological disease groups versus the reference group was compared. Significantly higher MBP values were observed in patients with multiple sclerosis (MS), cerebrovascular accident (CVA), tumour cerebri (adults), metabolic disorder and infection. Furthermore, significantly higher values were demonstrated for S-100 in CVA and for NSE in metabolic diseases, whereas significantly lower values were observed in communicating hydrocephalus. To determine a possible (in)dependent behaviour of these proteins we performed correlation analyses for 2 groups of patients with acute neurological disorder (MS and CVA). In CVA, the NSE and S-100 values were significantly related with MBP values, whereas in MS the NSE and S-100 were not related with MBP values. This might suggest that the demyelination process is different in MS compared to CVA.

## INTRODUCTION

Neuron-specific enolase (NSE), S-100 protein (S-100) and myelin basic protein (MBP) are regarded as nervous-system-specific proteins. NSE, the  $\gamma\gamma$ -isoenzyme of enolase, is a soluble cytoplasmic protein localized mainly in neurons [1]. S-100 constitutes a major component of the cytosol, predominantly in glial cells [2,3]. MBP is detectable in developing oligodendroglia and is bound to the cellular membranes of central and, to a lesser extent, peripheral myelin [4,5]. Thus, increased concentrations of NSE, S-100 and MBP in CSF may indicate CNS damage and may help to identify the cell type or part (neuron, glia or myelin) affected by the pathological process.

Over the past 10 years, a number of reports have appeared on abnormalities of brain-specific proteins in neurological patients. Recently, we reported that the concentrations of NSE, MBP and S-100 in the CSF of a reference group of adults and children, were age-dependent [6]. Therefore levels of CSF brain specific-proteins in neurological patients should be compared to age-related reference values. Most of the studies conducted until now have dealt with adults, so there is a lack of data in the literature on the appearance and significance of these markers in children with neurological disorders. Finally, most of the studies only measured one brain-specific protein and the findings and interpretations remain restricted to one brain compartment. This study described possible abnormalities of three brain compartments simultaneously; neurons, glial cells and myelin.

In this study we examined values of NSE, S-100 and MBP in the CSF of young and adult patients with distinct neurological disorders and compared the results to age-related reference values. We also investigated whether these brain-specific proteins behave independently and show distinct profiles in some neurological disorders.

## PATIENTS AND METHODS

### Patients

CSF samples were obtained from patients who underwent a diagnostic lumbar



puncture for a clinical indication, such as CNS infection or another neurological disorder. Lumbar puncture was generally performed within 4 days after admission to hospital. We obtained CSF samples from 596 patients with a definite diagnosis of an organic neurological disorder (357 adults and 239 children) referred to our Neurological Department between 1986 and 1990.

Children are different from adults with respect to the appearance and types of CNS involvement and were therefore studied separately. The patients (children and adults) were divided into several neurological subgroups (Table 1). In order to investigate whether brain-specific proteins behave independently, we examined the CSF patterns in two neurological disorders in more detail: multiple sclerosis (MS) and cerebrovascular accidents (CVA). Our study group comprised 91 MS patients with definite clinical MS according to Poser's criteria [7]. All the patients showed symptomatic deterioration of the disease (chronic progressive  $n=42$ , relapsing remittent  $n=34$  and a combination  $n=15$ ). The CVA patient group consisted of 41 non-haemorrhagic patients. It was not always possible to obtain an adequate CSF volume for all three protein examinations.

## Methods

CSF samples were centrifuged ( $1500 \times g$  for 10 min.) and a 0.5 ml portion of the cell-free supernatant was stored at  $-20^{\circ}\text{C}$  for the analysis of the brain-specific proteins. Any sample which showed definite blood contamination was discarded because human red cells contain a significant level of NSE [8]. The assay procedure for NSE, S-100 and MBP in CSF has already been reported previously [6]. In order to test for significant differences between the neurological groups and the reference group, the log transformed values were adjusted for the age trend and Dunnett's test for many-to-one comparisons was applied.

## RESULTS

After logarithmic transformation, a linear relation became clear in each group, with (the logarithm of) the concentrations generally distributed around a mean which

was directly proportional to age. In each group (with the possible exception of MBP in the MS group and the infantile encephalopathy group) the regression line through the means proved to run parallel to that for the reference group. Consequently, the mean value for each group and age differed from the mean value in the reference group by a constant amount. On the original (untransformed) scale this implies that (for each of the groups) the ratio between the median value (for that group) and the median value for the reference group was constant (independent of age). Therefore, it was possible to estimate the percentage increase or decrease of the (median) value for each group compared to the reference group, independent of age.

Table 1 presents the (age controlled) median value (as a percentage of the median in the reference group) of the three brain-specific proteins for the different patient groups. The percentage of patients with an abnormal value ( $> p95$  percentile) is given for each parameter in the various patient groups. For example, in the MS group, 40% of the patients had a MBP value which was higher than the  $p95$  of the reference group (age controlled).

The median NSE value in the hydrocephalus patients was significantly lower than that in the reference group, whereas it was significantly higher in the group with metabolic diseases ( $p < 0.05$ ).

The median MBP value in the patients with MS, CVA, tumour (adults), metabolic disease, infection, including infection in children, was significantly higher ( $p < 0.05$ ) than in the reference group.

The median S-100 value in the CVA group, was significantly higher ( $p < 0.05$ ) than in the reference group.

Table 2 presents the individual values of the 3 brain-specific proteins in the CSF of 9 patients with a metabolic neurological disorder. In the two Tay-Sachs patients the NSE level was strongly elevated. The MBP value was also increased in the infantile form, but normal in the juvenile form.

To determine whether the three protein markers behave (in)dependently, we performed correlation analyses (age-controlled) on the log values of two groups of patients with acute neurological disorders (MS and CVA) and the reference values. The results are presented in Table 3. In the CVA patients, the MBP values were

Table 1. Brain-specific proteins in various groups of neurological disorders.

patient group	NSE <sup>..</sup>			MBP			S-100		
	n	median (%)	> p95 (%)	n	median (%)	> p95 (%)	n	median (%)	> p95 (%)
<u>Adults:</u>									
Multiple sclerosis	91	90	12	90	215*	40	87	92	7
Dementia	15	97	0	14	109	7	16	101	12
Epilepsy (cryptogenic)	12	105	0	10	163	40	12	121	25
Head injury (mild)	10	121	0	9	162	33	10	105	0
Cerebrovascular accident(CVA)	39	119	13	37	403*	63	41	150*	22
Hydrocephalus (communicating)	10	56*	30(-)	9	174	22	10	98	20
Tumour cerebri (incl. 4 metastases)	11	110	19	10	302*	60	11	113	27
Hernia nuclei pulposa (HNP)	62	97	2	55	108	8	62	119	12
Infection	7	127	14	7	293*	43	7	162	43
Extra pyramidal disorder	26	94	4	25	97	0	26	82	0
<u>Children:</u>									
Epilepsy (cryptogenic)	32	102	6	68	132	18	33	96	21
Drain dysfunction	8	110	25	13	145	31	8	78	12
Congenital disorder	21	127	10	61	158	25	28	103	21
Infantile encephalopathy	19	90	5	48	129	27	19	108	21
Tumour cerebri	4	130	25	8	214	50	5	208	80
Neuromuscular disease	8	115	12	15	101	20	8	110	25
Migraine	2	119	0	11	208	45	5	198	60
Infection	6	124	0	7	322*	43	5	144	40
Metabolic neurological disorder	6	225*	60	9	296*	67	6	86	17

\* Significantly different from the control group (Dunnett's t-test,  $p < 0.05$ ).

.. Median (%): the (age controlled) median value (as a percentage of the median of the control group).

... p95 (%): the percentage of abnormal values (&gt; p95 percentile of the control group).

(-) p95 (%): the percentage of abnormal values (&lt; p5 percentile of the control group).

Table 2. Brain-specific proteins in the CSF of 9 patients with a metabolic neurological disorder.

Diagnosis	age (in years)	NSE μg/l	S-100 μg/l	MBP μg/l
Mitochondrial encephalomyopathy	0.2	9.0	1.8	1.0*
Mitochondrial encephalomyopathy	10	-	-	0.9*
Mitochondrial encephalomyopathy	1	-	-	0.4
Tay-Sachs (juvenile)	14	39*	3.3*	0.4
Tay-Sachs (infantile)	0.5	21*	1.6	18*
Ceroid lipofuscinosis	5.2	2.8	0.8	0.8*
Zellweger's syndrome	0.6	9.2	0.7	0.7
Adrenoleukodystrophy	5.5	-	-	3.0*
Nonketotic hyperglycinemia	0.1	11*	1.6	1.9*

\* = value > p95 of the age-related reference value

Table 3. Correlations between MBP and NSE, MBP and S-100, and between NSE and S-100 in 3 patient groups.

patient group	n	MBP/NSE R <sup>*</sup>	MBP/S-100 R	NSE/S-100 R
MS	86	0.01	0.15	0.31**
CVA	36	0.54**	0.45**	0.59**
Reference	47	0.05	0.05	0.15

\* R = correlation coefficient

\*\* Significant (p < 0.05)

significantly related with the NSE and S-100 values, whereas in the MS patients, the MBP values were not related with the NSE and S-100 values. We found only a minor relation between NSE and S-100 in the MS group and a clear relation in the CVA group. In the reference group, there was no significant relation between the three parameters. Figure 1 presents the individual MBP values in the CSF of the reference group, the MS group and the CVA group.

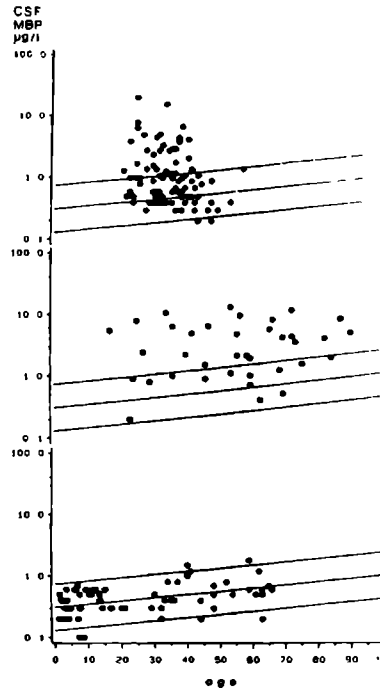


Figure 1. Scatter diagram of CSF MBP concentrations on a logarithmic scale as a function of age for reference patients (bottom), CVA patients (middle), MS patients (top). Solid lines indicate 5-95% confidence limits and median values of the reference patients.

## DISCUSSION

The diagnostic and prognostic value of CSF levels of the brain-specific proteins NSE, S-100 and MBP in neurological diseases have formed a frequent study topic. Increased CSF values of (some of) these brain-specific protein markers have been observed in: inflammatory diseases of the CNS [3,9-11], MS [3,12-15], CVA [2,9,11,17], head injuries [2,18], CNS tumours [4,9-11,19,20], Creutzfeld-Jakob's disease [21], epilepsy [11,22] and other neurological disorders.

In our study population, we observed significantly increased MBP values in six different patient groups: in MS, CVA, tumour cerebri (adults), infection (adults and children) and metabolic disorders.

### *Multiple sclerosis*

We observed increased MBP levels in the patients with the chronic progressive form and in those with the relapsing remittent form. All the patients had an active form of the disease. Several authors have reported on the appearance of increased CSF MBP levels in patients with active MS [12,15,24,25]. In a previous study performed at our department on the same MS group, we stated that there was a correlation between the CSF MBP level and the clinical score on the EDSS (Kurtzke's expanded disability status scale) of relapsing MS patients [14]. Martin-Mondiere [26] only observed increased CSF MBP levels in MS patients with an exacerbating course with new lesions. He found normal CSF values both in active chronic progressive MS and in exacerbating MS with recurrence of old signs or symptoms. The levels of NSE and S-100 in our study were no different from the control values.

### *Cerebro-vascular accident*

We found increased MBP levels in ischemic disorders of the CNS, which is in agreement with the results published in other reports [12,19] and indicate that demyelination often occurs in association with these disorders. According to Whitaker et al [25] the degree of MBP elevation generally correlates with the volume of the lesion. In our patients, the S-100 level was also significantly increased and indicated concomitant parenchymal damage of the CNS; this is in accordance with other reports [3,9,17,27,28].

The low incidence of elevated NSE in the CVA patients in our study was not surprising, because neuronal damage occurs predominantly in severe stroke patients where the infarct involves both the cerebral cortex and the white matter [9]. In a recent study performed at our neurological department on 8 patients who had suffered severe infarction of the middle artery, we observed a clearly increased CSF NSE value in all of them (unpublished data).

### *Tumours*

We found increased MBP levels in our patients with primary brain tumours, but

not in the patients with brain metastases. Raised MBP levels have also been demonstrated by other authors in patients with leptomeningeal metastases [5] and in primary intracranial tumours [19]. Increased S-100 levels were found frequently in our patients, while the NSE values were unchanged with the exception of a very high CSF NSE level in a patient with a cerebellar tumour. Increased NSE values have been reported in the literature, especially in primary tumours [11,20].

### *Infections*

In young and adult patients with an infection of the CNS we found significantly elevated MBP levels. The S-100 levels were also increased ( $\geq 40\%$ ) but not significantly, while the NSE values remained within the normal range in both age groups. Several studies have appeared on brain-specific proteins in infections of the CNS. Mokuno et al [9] observed significantly elevated S-100 levels in meningitis and encephalitis, while the NSE values were only increased in encephalitis.

### *Metabolic disorders*

In our patients with a metabolic neurological disorder, an increased MBP level occurred very frequently, e.g. in infantile Tay-Sachs, non-ketotic hyperglycinemia, adrenoleukodystrophy, ceroid lipofuscinosis and mitochondrial encephalomyopathy (Table 2). It is remarkable that the CSF MBP was normal in juvenile Tay-Sachs and abnormal in the infantile form. It is known that in severe infantile cases there is enormous accumulation of G<sub>M2</sub>-gangliosides in neurons which may amount to 12 per cent of the dry brain weight. Such an accumulation of storage products may lead to other secondary aberrations, e.g. demyelination. In Tay-Sachs with a late onset, the G<sub>M2</sub> accumulation is far less pronounced.

The NSE values in both Tay-Sachs patients were strongly increased, which indicates neuronal damage as a consequence of gangliosides storage.

### *Communicating hydrocephalus*

In our communicating hydrocephalus group, the significantly decreased CSF NSE level was striking. We could not find any data on these markers in the literature. The

normal levels for MBP and S-100 in the presence of a decreased NSE, could indicate loss or atrophy of neurons in this disorder.

### *Other disorders*

In our patient groups, various disorders were associated with (non-significantly) increased CSF brain specific protein values. Increased MBP levels ( $> p95$ ) in the absence of S-100 and NSE elevations were observed in the CSF of adults and children with neurological disorders, such as trauma capitis and drain dysfunction. Increased MBP and S-100 levels were observed in patients with epilepsy and migraine. Our patients with HNP, dementia or an extrapyramidal disorder, did not show abnormal levels for the three proteins. In a recent study conducted at our department on 135 patients with various types of dementia, the CSF brain specific proteins were also normal (unpublished data).

### **Comparison of two acute neurological disorders**

We observed different patterns of the brain-specific proteins in the CSF of two patient groups with acute neurological disorders: one group had deteriorating MS and the other group had suffered a CVA. Lumbar puncture was always performed within 4 days after admission to hospital.

In our MS patients we found increased MBP values and normal levels for NSE and S-100. The absence of NSE elevation may indicate that neurons are not involved in the acute stage of the disease.

Conflicting results have been reported for S-100 in MS patients. Michetti et al. [29,30] found increased CSF S-100 levels in patients with active MS and interpreted them as an index for active cell injury during exacerbation of the disease. Our results support those reported by Noppe et al. [27] who did not find any S-100 abnormalities in patients with active MS. Massaro [13,31] demonstrated that MBP and S-100 were not released simultaneously following exacerbation. MBP reached its highest level during the first week, while S-100 reached its peak during the third week. This suggests that the origin of S-100 and its pathobiological significance in MS are different from those of MBP. In addition, S-100 cannot be regarded as a direct



marker of myelin destruction in MS, but is more likely to indicate the onset of gliosis in the CNS or increased astrocytic secretory activity.

In our CVA patients, both the S-100 and MBP values were significantly increased and a correlation was found between them, in contrast to those in MS. This might indicate that (parenchymal) astrocytic involvement is associated with demyelination in CVA. It is likely that demyelination in CVA is secondary to other causes while demyelination in MS is a primary process.

### **General remarks**

- We should be very cautious about drawing conclusions about the levels of CSF brain-specific proteins in patient groups and individual patients. The levels of brain-specific proteins in CSF depend on several factors, such as the distance between the affected brain area and the CSF compartment, the severity and extent of brain damage, the regional variability of these proteins in the brain, the degradation of these proteins by macrophages and/or proteinases either locally or in the CSF.

Also the time of lumbar puncture in relation to the onset of tissue damage is a very important factor. It is known that an increase in the levels of these proteins in the CSF is maintained for at least 4 to 8 days post-damage, depending on the type, extent and location of the brain lesion. In this study we used existing data without any well-defined standardization of the CSF sampling time. However, our records showed that lumbar puncture was generally performed within 4 days after admission to hospital.

- Increased levels of these proteins in CSF indicate that the brain compartment is affected. However, normal levels of (one of) these proteins does not exclude the presence of local brain damage.

- We observed significantly increased brain-specific protein levels in various patient groups and non-significant increases in other groups. It is possible that the numbers of patients in some of these groups were too small to reach statistical significance, for example the MBP and S-100 values in children with tumour cerebri.

- Increased values for CSF MBP were observed most frequently, S-100 less frequently and NSE least frequently. The frequent occurrence of an increase in MBP can partly be explained by the fact that the regions of the brain close to the ventricles consist

mainly of myelinated white substances. NSE is released from the grey substance of the cortex which is further away from the ventricles and it therefore takes longer for it to appear in CSF. It is also possible that a proportion of NSE leakage is transferred from the extracellular space directly via capillary wells into the blood stream and does not enter the CSF space. This might explain the rapid NSE increase in the serum of the patients in coma after head injuries [23]. Finally, neurons might be more resistant against damage than myelin.

## CONCLUSION

Increased CSF levels of brain-specific proteins are found in patients who are suffering from brain tissue damage. This damage can be the result of 1) an acute neurological disorder with brain damage, such as CVA, exacerbating MS and infection or 2) a chronic progressive disorder, such as active chronic progressive MS, metabolic disorders (Tay-Sachs) and tumour cerebri.

Data on brain-specific proteins in CSF provide information on the pathophysiology of a particular disease process. It is likely that the concentration of these proteins in CSF might be of relevance for assessing the severity and the activity of a disease process and for monitoring treatment effects.

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## **CHAPTER 4, section 3**

### **THE SHORT-TERM EFFECT OF AN IMMUNOSUPPRESSIVE TREATMENT ON CEREBROSPINAL FLUID MYELIN BASIC PROTEIN IN CHRONIC PROGRESSIVE MULTIPLE SCLEROSIS**

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## SUMMARY

Cerebrospinal fluid (CSF) levels of myelin basic protein (MBP) and intrathecally produced CSF IgG (de novo IgG) were measured in 11 chronic progressive multiple sclerosis patients with a deteriorating course of the disease for at least 6 months preceding observation and a reference group of 17 neurological patients suffering from disc herniation. In the multiple sclerosis patients, CSF levels were determined just before and once in the period 3 to 10 weeks after the start of an immunosuppressive treatment with cyclophosphamide and prednisone. For multiple sclerosis patients the CSF MBP levels before treatment were significantly higher than for controls. The CSF MBP levels after the treatment were nearly all within the control range. The high concentration of intrathecally produced CSF IgG (de novo IgG) in multiple sclerosis patients was reduced after treatment. A correlation between CSF MBP and CSF de novo IgG in multiple sclerosis patients could not be demonstrated. If CSF MBP is an indicator of the myelin breakdown in the brain, it can be concluded that an intensive immunosuppressive treatment in combination with prednisone has, at least, a short-term, beneficial effect on the amount of demyelination and possibly on the disease activity.

## INTRODUCTION

Increased levels of myelin basic protein (MBP) or fragments of it in cerebrospinal fluid (CSF) have regularly been observed in patients with neurological diseases such as head injury and stroke [1-7]. The measurement of MBP in CSF has appeared to be a useful indicator of tissue damage. Furthermore, recent studies in multiple sclerosis patients have also shown increased MBP levels in CSF, especially after an acute exacerbation of the disease [3,4,8-12]. From these studies one may conclude that the amount of CSF MBP in exacerbating forms of multiple sclerosis is higher (1) during the first week after onset of an attack [2,11], (2) in polysymptomatic attacks compared with monosymptomatic attacks [10], (3) in severe attacks with complete paralysis [4] and (4) during exacerbations with the presence of new signs and symptoms [12]. In chronic progressive forms of multiple sclerosis increased MBP levels in CSF are regularly found, but the percentages of abnormalities and the amount of MBP in CSF are generally lower than during an acute phase of the disease process in exacerbating forms [3,4,10,12]. If the CSF MBP levels can be used as an indicator of demyelination and/or disease activity in patients with multiple sclerosis, measurements of CSF MBP before and after therapy might give information about the effect of the therapy on the myelin degradation in the central nervous system (CNS).

The purpose of our investigation was to study the possible effect of an immunosuppressive treatment on the level of CSF MBP in 11 chronic progressive multiple sclerosis patients with a deteriorating form of the disease. The effect of treatment on intracerebrally produced IgG and the relation between IgG and MBP were also studied.

## SUBJECTS AND METHODS

### Subjects

The multiple sclerosis patients (n=11, age 20-36 yr) were definite multiple sclerosis patients with a chronic progressive course of the disease. These patients had a continuous deterioration of clinical symptoms for at least 6 months before treat-



ment. They received an intensive immunosuppressive treatment consisting of a daily dose of 400 mg cyclophosphamide and 100 mg prednisone, with a total dose of 8 g cyclophosphamide [13]. The duration of treatment was between 4 and 5 weeks. Just before starting treatment and once 3-10 weeks later blood and CSF samples were taken. The multiple sclerosis patients had never received any immunosuppressive treatment before. As reference a control group of neurological patients (n=17, age 24-72 yrs) suffering from disc herniation in whom no clinical signs of demyelination could be observed was used.

## Methods

### *MBP assay*

MBP was determined according to the instructions of the manufacturer, Diagnostic Systems Laboratories, Webster Texas USA cat. nr. 1500, using a double antibody RIA kit containing human myelin basic protein (whole molecule) as antigen and rabbit antihuman MBP as antiserum. Goat anti-rabbit gamma globulin was used as MBP precipitation reagent. Human MBP ( $J^{125}$  2 $\mu$ Ci/vial) was included in the test kit.

### *IgG/albumin assay*

IgG and albumin were measured in unconcentrated CSF and diluted serum (1:400). Sheep antihuman IgG (Boehringer, Mannheim) and rabbit antihuman albumin (Behringwerke, Marburg) were used in the assay.

$$Q \text{ alb} = \text{CSF alb/serum alb}$$

$$Q \text{ IgG} = \text{CSF IgG/serum IgG}$$

$$\text{IgG index} = Q \text{ IgG}/Q \text{ alb}$$

The calculation of CSF IgG, synthesized within the CNS (de novo CSF IgG), was performed according to the formula of Reiber [14] with minor modifications. De novo CSF IgG is total CSF IgG minus transsudative (serum derived) IgG.

### *Statistics*

For each of the biochemical variables we present the median value and the range of the observed levels. For the multiple sclerosis patients the statistical comparison of

the pre- and posttreatment values was performed by means of the Wilcoxon test for the paired case. The statistical comparison between different patient groups was performed by the Wilcoxon test for the unpaired case. The results of a comparison were considered to be significant if its corresponding p value was smaller or equal to 0.05. The statistical dependence between two variables was evaluated by means of Kendall's test for rank correlation.

## RESULTS

### *MBP*

The individual results of the CSF MBP determination are shown in figure I. The median value and the range of CSF MBP are given in table I. Within the group of 11 multiple sclerosis nine patients showed an elevated CSF MBP level (higher than the estimate of the 95th percentile for the control group = 2.3  $\mu\text{g/l}$ ) before treatment. After treatment only three patients had increased levels of CSF MBP.

For multiple sclerosis patients the CSF MBP levels were significantly ( $p = 0.01$ ) lower after treatment (median 1.7  $\mu\text{g/l}$ ) than before treatment (median 4.2  $\mu\text{g/l}$ ). The MBP levels for multiple sclerosis patients before treatment were significantly higher than for controls ( $p = 0.001$ ), while the MBP levels of multiple sclerosis patients after treatment were not significantly different from that of controls ( $p = 0.29$ ). From these results it can be concluded that the abnormal CSF MBP levels of "active" chronic multiple sclerosis patients are decreased to the reference limits 3-10 weeks after the start of the treatment.

### *De novo CSF IgG*

The median values and ranges for CSF de novo IgG and IgG index in multiple sclerosis patients and controls are presented in table I.

With respect to CSF IgG, before treatment all multiple sclerosis patients had increased IgG indices (higher than the upper limit (0.59) of reference values from our laboratory) and de novo CSF IgG production (median 43  $\text{mg/l}$ ).

After treatment, the de novo CSF IgG value (median 19  $\text{mg/l}$ ) was significantly ( $p = 0.004$ ) decreased compared to the pretreatment value.

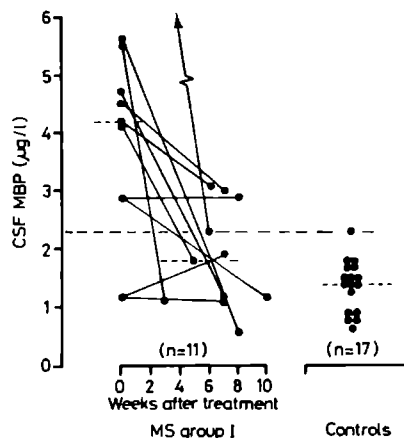


Figure 1: CSF MBP values in chronic progressive multiple sclerosis patients with a deteriorating course before and after treatment and controls.

#### *Relation between MBP level and de novo IgG level in CSF*

We could not demonstrate any significant correlation between MBP and de novo IgG or IgG index in the multiple sclerosis group.

Table 1: Median value and range for CSF MBP, de novo IgG and IgG index in multiple sclerosis patients and controls.

Patients	Number	Before treatment		After treatment		Comparison p-value
		Median	Range	Median	Range	
		MBP in CSF (µg/l)		MBP in CSF (µg/l)		
Multiple sclerosis group	11	4.2	(1.2-15)	1.7	(0.6-3.1)	0.01
Controls	17	1.4	(0.7-2.3)			
		De novo CSF IgG (mg/l)		De novo CSF IgG (mg/l)		
Multiple sclerosis group	11	43	(16-100)	19	(0-70)	0.004
		IgG index		IgG index		
Multiple sclerosis group	11	1.25	(0.93-3.21)	1.02	(0.76-1.91)	0.002

## DISCUSSION

CSF MBP levels are probably an indication of the degree of demyelination in multiple sclerosis patients and its quantitation might be used to monitor disease activity and to study the effect of putative therapy. The actual level of CSF MBP reflects the amount of tissue undergoing demyelination, the actual rate of myelin breakdown and the location of the lesion with respect to the lumbar space [4]

In exacerbating forms of multiple sclerosis it has been emphasized that CSF MBP levels mostly return to normal values 2 to 4 weeks after acute attacks [2-4,9]. This, however, is not the case in patients in whom clinical deterioration still remains after recovery [4,11].

Elevated CSF MBP levels are only found in chronic progressive multiple sclerosis patients with a deteriorating course [3,4,12]. The results of our study confirm abnormal CSF MBP values in deteriorating chronic progressive multiple sclerosis patients.

After intensive immunosuppressive treatment CSF MBP levels normalise in nearly all patients. The CSF MBP levels in the multiple sclerosis patients, averaged 6 weeks after treatment, can statistically not be distinguished from controls. If CSF MBP is an indicator of myelin breakdown in the brain, one can conclude that an intensive immunosuppressive treatment in combination with prednisone has, at least a short-term, beneficial effect on demyelination and possibly on the disease activity. The question can be raised whether the effect of treatment on disease activity can be measured by its alterations in CSF MBP levels.

With respect to the IgG, this study shows that all multiple sclerosis patients had both an intrathecally produced CSF IgG before treatment and a clear reduction of the de novo IgG amount after treatment. Although treatment has a beneficial effect on the amount of intrathecal IgG production, the de novo IgG production was not stopped.

Finally, the absence of a correlation between CSF MBP level and de novo IgG production might suggest that demyelination and (local) immunological activity of IgG are not related. A relationship between disease activity and intrathecally IgG production is rarely observed in multiple sclerosis studies.

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## **CHAPTER 4, section 4**

### **A CORRELATIVE TRIAD OF GADOLINIUM-DTPA MRI, EDSS AND CSF-MBP IN RELAPSING MULTIPLE SCLEROSIS PATIENTS TREATED WITH HIGH- DOSE INTRAVENOUS METHYLPREDNISOLONE**

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## SUMMARY

In a prospective study, we compared the number of gadolinium-DTPA (Gd-DTPA) enhancing lesions on MRI with the cerebrospinal fluid (CSF) and clinical findings before and after a total of 20 courses of high-dose intravenous methylprednisolone (MP) in relapsing multiple sclerosis patients. Before treatment, there was a significant correlation of Gd-DTPA enhancement (seen on 16 of 20 scans) and CSF myelin basic protein (MBP). If enhancement with Gd-DTPA represents inflammation and CSF-MBP indicates myelin breakdown, the amount of inflamed tissue should correlate with the amount of myelin being damaged. Clinical improvement occurred following 15 to 20 courses, and decrease of Gd-DTPA enhancement in 12 of 16 scans; the mean CSF-MBP level was the only CSF variable to return to reference values. There was a significant correlative triad of decrease in CSF-MBP, Gd-DTPA enhancement, and clinical disability. Thus, the clinical effect of methylprednisolone might be accompanied by a reduction of inflammation and myelin breakdown.

## INTRODUCTION

Blood brain barrier (BBB) disruption is an early [Grossman 1988, Miller 1988, Bastianello 1990] or even preceding [Kermode 1990] feature in the acute stage of lesion development in MS. BBB disruption can be demonstrated by means of Gd using MR imaging. A recent study showed that high-dose IV MP improved BBB integrity in MS, as judged from changes in Gd-enhancement [Barkhof 1991]. Improved BBB integrity was found to correlate with clinical improvement.

CSF examination yields typical abnormalities in MS patients, which reflect the immunological status of the patient. After MP treatment, changes in CSF variables occur, which consist of reduction of MBP [Frequin 1990, Warren 1986], anti-MBP [Warren 1986, Wajgt 1983], and reduction of intrathecal IgG-synthesis [Frequin 1990, Warren 1986, Durelli 1986, Milligan 1987]; a reduction in the number of oligoclonal gamma bands (OB's) with iso-electric focusing has been found by Frequin [1991], but not by Durelli [1986]. There is no clear correlation between CSF and clinical findings, except for CSF-MBP which is raised at times of relapse [Cohen 1980, Martin-Mondiere 1987] and is undetectable in the remission phase [Warren 1987].

We hypothesize that there is a correlation between Gd-enhancement and some CSF variables, as they all express activity on an immunological level. The purpose of the present study is to investigate a) whether there is a relation between clinical findings, Gd-enhanced MR imaging and CSF variables in relapsing MS patients before treatment and b) whether MP-induced changes in clinical findings and Gd-enhanced MR imaging relate to changes in CSF variables.

## PATIENTS AND METHODS

Sixteen patients (12 women, 4 men) with definite MS [Poser 1983], were prospectively investigated. The mean age of the patients was 34 years (range, 22 to 45), the mean DD 6.8 years (range, 1 to 15). All the patients were treated because of a symptomatic deterioration lasting longer than 24 hours, without subsequent spontaneous improvement. Treatment consisted of IV MP administered during 15



minutes in a dose of 1 gram daily for 10 consecutive days. Four patients were treated twice (interval at least 3 months). A total of 20 consecutive courses was thus given and used for analysis. Spinal taps and MR scans were performed before and after MP treatment. The mean interval between the first tap and first MR scan was 8.6 days (range, 0 to 22); the mean interval between the second tap and second MR scan was 4.2 days (range, 0 to 14). EDSS-scores [Kurtzke 1983] were assessed before and after therapy simultaneously with the CSF examination, by an examiner (ORH) who was blinded to the MR and CSF findings.

MR imaging was performed on at 0.6 Tesla system (Technicare, Solon, Ohio) before and after treatment using a standard head coil. Repositioning errors were corrected by means of 3 consecutive oblique scout images (transverse, coronal, sagittal). Gd-DTPA dimeglumine (Schering AG, Berlin) was administered IV in a dose of 0.2 mmol/kg, followed by a post-injection flush with 10 cc saline. Axial (double oblique) series were made according to internal landmarks (caudal borders of the pituitary gland and the fastigium of the 4th ventricle for angulation; caudal border of the splenium of the corpus callosum as Z-centre). T<sub>1</sub>-weighted SE sequences were obtained (repetition time 400 ms/echo time 28 ms/4 excitations) starting 5-10 minutes after Gd-injection, with an in-plane resolution of 1.0 x 1.3 mm, and a slice thickness of 5 mm (gap 1.25 mm). MR scans were analyzed by 2 of the authors (FB, PS) in conference; they were unaware of the clinical and CSF findings. An area of markedly increased SI, not related to a physiologically enhancing structure, and consisting of at least 3 pixels, was considered a Gd-enhancing lesion. The number, not the size, of Gd-enhancing lesions was used as study-parameter.

CSF examination was performed before and after treatment. CSF analysis included mononuclear cell count, albumin-quotient (Q-albumin), intrathecal IgG-synthesis, the number of OB's on iso-electric focusing and CSF-MBP. IgG and albumin were measured in unconcentrated CSF and diluted serum (1:400). Sheep antihuman IgG (Boehringer, Mannheim) and rabbit antihuman albumin (Behringwerke, Marburg) were used. Q-albumin was defined as albumin-CSF/albumin-serum. The calculation of intrathecal IgG-synthesis (intrathecally produced IgG) was performed according to the hyperbolic Reiber formula [Reiber 1987]. Iso-electric

focusing was performed with Ampholine PAG-plates (by LKB; pH 3.5-9.5). MBP was determined according to the instructions of the manufacturer (Diagnostic Systems Laboratories, Webster, Texas) using a double antibody RIA-kit containing human basic protein (whole molecule) as antigen and rabbit anti-human MBP as anti-serum. Goat anti-rabbit gamma-globulin was used as MBP precipitation reagent. Human MBP ( $J^{125}$  2  $\mu$ Ci/vial) was included in the test-kit.

Statistical analysis was performed using the SPSS/PC<sup>TM</sup> statistical package. Data before and after treatment were compared by means of a Wilcoxon matched-pairs signed-rank test. Relations between clinical, MR and CSF data were investigated by means of Pearson's correlation coefficients. Nullhypotheses were tested two-sided and p-values smaller than 0.05 were considered statistically significant; p-values between 0.05 and 0.10 were considered as trends.

## RESULTS

Before MP-treatment, the median EDSS-score was 3.5 (mean 4.2), 84 Gd-enhancing lesions were seen in 16 out of 20 scans (4 scans showed no enhancement) and all the mean values of the investigated CSF variables, except Q-albumin, were abnormal. Following treatment a reduction in EDSS score (median, 1.0) was observed (after 15 of 20 courses improvement was seen). Table 1 shows the mean values of clinical, CSF, and MR findings before and after treatment. Insignificant reduction in mononuclear cells (25%), OB's (6%), and Q-albumin (18%) were found after MP-treatment. Following treatment there was a significant reduction in the number of Gd-enhancing lesions (76%; 3 out of 16 scans showed less enhancement and 9 out of 16 scans showed no enhancement), intrathecal IgG synthesis (66%), and CSF-MBP level (74%). After treatment, only the mean CSF-MBP level returned to reference values, while the mean values of all the other CSF variables investigated remained elevated. Table 2 displays the initial EDSS-scores, and changes after MP-treatment in EDSS, Gd-enhancement and CSF-MBP for the individual patients.

The relation between Gd-enhancement, EDSS-score and CSF variables before treatment is shown in table 3. Only the number of Gd- enhancing lesions and the

CSF-MBP level correlated significantly. Trends were noted in the relation of Q-albumin to Gd and in the relation of CSF-MBP to EDSS. There were no correlations of age or DD with either the EDSS-score or the number of Gd-enhancing lesions before treatment (not shown in table).

The relation between the change in EDSS score and number of Gd-enhancing lesions on the one hand and change in CSF variables on the other hand is shown in Table 3. Significant relations were found in the decrease in number of Gd-enhancing lesions to the decrease in EDSS-score and in the relation of decrease in CSF-MBP level to both decrease in EDSS-score and decrease in the number of Gd-enhancing lesions. A trend was noted towards a negative correlation of the decrease in number of Gd-enhancing lesions and the change in number of OB's. There was a significant relation between the difference in EDSS score with the initial EDSS score ( $r=0.631$ ,  $p=0.003$ ) and with age ( $r=-0.464$ ,  $p=0.039$ ), and a trend in the difference in number of Gd-enhancing lesions with either initial EDSS-score ( $r=0.398$ ,  $p=0.08$ ) or age ( $r=-0.390$ ,  $p=0.09$ ). The DD did not correlate with the difference in both EDSS-score or the number of Gd-enhancing lesions (not shown in table).

Table 1: Median EDSS and mean MR and CSF variables before and after high-dose intravenous MP treatment.

variable	reference	before (range)	SD	after (range)	SD	p-value
EDSS score	0	3.5 (2-7)	1.3	2.25 (2-6)	1.2	0.0007
Gd-lesions	0	4.2 (0-33)	7.9	1.0 (0-14)	3.1	0.0037
mono's (mm <sup>3</sup> )	< 9/3	17.4 (0.78)	16.3	13.0 (2-49)	11.9	NS
IgG synth. (mg/l)	0	23.6 (0-135)	32.8	8.1 (0-34)	9.0	0.0053
number of OB's	0	3.4 (0-12)	3.2	3.2 (0-13)	3.3	NS
CSF-MBP (µg/l)	≤ 1.5	2.3 (0-11.6)	3.3	0.6 (0.2-2.5)	0.5	0.0042
Q-alb (x10 <sup>-3</sup> )	< 670	612 (264-1,217)	217	578 (296-1,191)	227	NS

p-values were calculated using Wilcoxon's matched pairs signed rank test. SD: standard deviation; NS: not significant; mono's: number of mononuclear cells; IgG synth.: intrathecal IgG synthesis; Q-alb: albumin quotient.

Table 2: Clinical data and treatment effect on EDSS, Gd, and CSF-MBP for each patient.

patient	age	sex	DD	entry-EDSS	$\Delta$ -EDSS	$\Delta$ -Gd	$\Delta$ -CSF-MBP
A	35	M	15	3.0	1.0	0	0.70
B	34	F	1.5	3.0	1.0	0	-0.30
C	39	F	15	4.0	2.0	0	5.30
D	30	F	4	6.0	0	4	0.30
E1	26	M	1.5	5.0	2.0	19	4.10
E2				7.0	5.0	17	5.50
F1	39	F	2.5	3.0	0	0	0
F2				3.0	1.0	0	-0.30
G1	33	F	2.5	4.0	1.5	0	0
G2				3.0	1.0	1	0.80
H1	27	F	3	2.0	0	1	-0.10
H2				3.0	1.0	1	0.10
I	22	F	1.5	3.0	1.0	1	0.10
J	28	F	3.5	6.0	2.0	5	1.30
K	35	F	5	3.0	0	10	7.40
L	36	M	6	4.0	1.0	-1	0.30
M	27	F	4.5	5.0	2.0	0	0.20
N	45	F	24	7.0	1.0	1	0.30
O	39	F	10	3.0	0	1	0
P	29	M	10	6.0	3.0	3	9.10

$\Delta$ -EDSS,  $\Delta$ -Gd,  $\Delta$ -CSF-MBP: change in EDSS score, number of Gd-lesions and CSF-MBP level following treatment.

Table 3. Relation between EDSS scores, Gd-enhancement and CSF variables, expressed as Pearson's correlation coefficients.

	Number of Gd-lesions		EDSS-score	
	before treatment	treatment effect	before treatment	treatment effect
Gd-lesions			0.13	0.50 (p=0.03)
mono's	0.12	0.13	-0.06	0.00
intrathecal IgG	-0.05	-0.16	0.23	0.02
OB's	-0.27	-0.44 (p=0.05)	0.12	-0.36
CSF-MBP	0.46 (p=0.04)	0.56 (p=0.01)	0.39 (p=0.09)	0.52 (p=0.02)
Q albumin	0.40 (p=0.09)	0.37	0.32	0.36

The Pearson's correlation coefficients shown are not statistically significant unless otherwise indicated (p values).

## DISCUSSION

MR imaging is a very sensitive tool in the assessment of MS lesions [Paty 1988, Uhlenbrock 1988, Baumhefner 1990]. Lesions on T<sub>2</sub>-weighted images can not

differentiate the separate pathological stages of lesion development and represent both active and inactive disease. The lesion load on T<sub>2</sub>-weighted MR imaging represents predominantly disease activity in the past and is subject to small fluctuations only; some have found these to correlate with the EDSS-score [Truyen 1990] and with neuropsychological testing [Baumhefner 1990, Rao 1989]. The number of hyperintense foci on T<sub>2</sub>-weighted images has previously been found to correlate with intrathecal IgG-synthesis [Baumhefner 1990, Baum 1990], but not with the number of mononuclear cells [Baum 1990] or OB's [Baumhefner 1990]. The relation of CSF-MBP to the number of hyperintense foci has never been investigated. MR imaging is capable of separating lesions with and without BBB disruption by means of Gd. The relation of Gd-enhancement to CSF findings has, to our knowledge, never been investigated.

Gd-enhanced MR imaging and some CSF variables present information about the separate (early) stages of lesion development or disease activity at present and are subject to major fluctuations [Grossman 1988, Miller 1988, Bastianello 1990, Warren 1987, Thompson 1983 and Thompson 1987]. We hypothesized that there would be a relation between Gd-enhancement and (some) CSF variables. Before treatment a significant relation was found between the number of Gd-enhancing lesions and the level of CSF-MBP. If Gd-enhancement stands for active inflammation [Katz 1990, Hawkins 1991, Kuharik 1988] and CSF-MBP stands for demyelination [Warren 1987], the volume of inflamed tissue relates to the amount of myelin being damaged and indicates that inflammation is directly accompanied (and not followed) by demyelination. Evidence for the occurrence of demyelination early in the development of lesions is also found in the demonstration of lipid peaks in the MR spectra of new lesions [Wolinsky 1990].

The intrathecal IgG synthesis is usually elevated during the remission phase of MS [Thompson 1987, Caroscio 1986] and therefore seems not especially linked to current disease activity. This might explain the correlation reported between intrathecal IgG-synthesis and T<sub>2</sub>-weighted MR imaging [Baumhefner 1990, Baum 1990] and the absence in our study of a correlation between intrathecal IgG-synthesis and Gd-enhancement. The number of OB's, although not constant over time [Thompson

1983], is not subject to great variations [Thompson 1983 and Thompson 1987], and the absence of a correlation with Gd-enhancement is therefore not surprising.

The rationale for immunosuppressive treatment, such as MP-treatment, is to suppress the inflammatory reaction of the immune system, in order to prevent or stop the process of demyelination [Warren 1987, Thompson 1989]. In the present study a significant decrease of intrathecal IgG-synthesis [Frequin 1990, Warren 1987, Durelli 1986, Milligan 1987] and CSF-MBP levels was found [Frequin 1990, Warren 1987] after MP-treatment, but not of OB's [Wajgt 1983], mononuclear cells number or Q-albumin. The CSF-MBP level, however, was the only CSF variable to return to reference values. Interestingly, the decrease in CSF-MBP level correlated significantly with the decrease in number of Gd-enhancing lesions and EDSS-score. After high-dose intravenous MP treatment both Gd-enhancement and CSF-MBP levels improved in a similar way, which might indicate that its clinical action is accompanied by improved BBB integrity and decreased myelin breakdown. The changes in other CSF variables did not correlate with the decrease in Gd-enhancement.

There was a trend towards correlation of the number of Gd-enhancing lesions and the Q-albumin before treatment, but the decrease in number of Gd-enhancing lesions following MP-treatment showed no significant relation with the changes in albumin-quotient (not significantly lower), although both represent BBB-integrity. In animal studies of experimental allergic encephalitis enhancement of Gd-albumin complex was only seen in 4 out of 6 Gd-enhancing lesions, while in 2 of these lesions the extent and intensity was less than with Gd [Hawkins 1990]. The molecular weight of Gd (550 Da) and albumin (60kDa) is considerably different, as might their routes of transport across the BBB be, thereby giving a possible explanation for the absence of a good correlation.

The correlative triad of clinical improvement, restoration of BBB-integrity and decrease of myelin breakdown following MP-treatment is certainly one of the most salient findings of this study. The decrease in recent lesions after MP is accompanied by a decrease in myelin breakdown. The changes in EDSS, Gd, and CSF-MBP could have been the result of the natural course. We think, however, that the finding of a correlative triad pleads against this. Placebo-controlled studies could clarify this issue.

If the changes in Gd-enhancement and CSF-MBP are indeed the result of MP treatment it is impossible to decide from these data whether they are a primary result, i.e. that MP actually interferes with the process responsible for demyelination, or that they are a secondary result, i.e., that MP only interferes nonspecifically with the mediators of the disease process or acts directly on the BBB and in that way prevents the transfer of mononuclear cells into the brain [Long 1985]. The fact that the number of mononuclear cells and OB's did not drop significantly might indicate that the inflammatory process is not completely stopped after MP treatment (or that both variables are not correlated with acute inflammatory activity in the brain).

Both age and initial EDSS score showed a significant correlation with the change in EDSS after treatment, but not with the decrease in Gd-enhancement, which might indicate that there was some favourable change after treatment (decreased BBB disruption), which was not appreciated by the EDSS scoring system in relatively older or less disabled patients. Clinical disease activity as assessed by the EDSS mainly represents the result of lesions in the brainstem and spinal cord. Serial MR studies have shown that MR activity is far more frequent (especially in so-called "silent regions") than clinical activity. It is therefore far from surprising that there is no perfect correlation between Gd-enhancement and EDSS changes.

The finding of a correlative triad of (changes in) CSF-MBP, Gd-enhancement and EDSS (following MP-treatment), indicates that these variables might become of more importance for the evaluation of progression of the disease and for monitoring clinical trials in addition to ordinal clinical scoring systems like the EDSS with their fallacious pitfalls [Noseworthy 1990, Francis 1991]. When substantiated this could have implications for the population size and follow-up time needed to be able to demonstrate a certain effect of putative drugs in treatment trials in MS.

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## **CHAPTER 5, section 1**

### **CEREBROSPINAL FLUID MYELIN BASIC PROTEIN AND IMMUNOGLOBULIN LEVELS IN 101 MULTIPLE SCLEROSIS PATIENTS BEFORE AND AFTER TREATMENT WITH HIGH-DOSE INTRAVENOUS METHYLPREDNISOLONE**

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## SUMMARY

A total of 101 patients (62 females; 39 males) with definite MS were treated with 1000 mgr methylprednisolone (MP) intravenously for 10 consecutive days. Immediately before and after MP treatment clinical scoring: Kurtzke's Expanded Disability Status Scale (EDSS) and cerebrospinal fluid (CSF) analysis were performed.

Before MP treatment CSF myelin basic protein (MBP), IgG and IgM levels immunoglobulin levels (CSF Ig, index and intrathecal synthesis) were significantly elevated. The mean CSF MBP levels were significantly higher in the relapsing-remitting (RR) and chronic progressive MS patients with relapses (CP+RR) than in the CP group without a RR disease course, respectively 2.1, 2.3 and 1.5  $\mu\text{g/l}$ . A weak positive correlation was found between CSF MBP level and EDSS in the RR MS group ( $r=.34$ ). CSF MBP was significantly correlated with IgM index ( $r=.36$ ), IgM synthesis ( $r=.26$ ), but not with the IgG levels. Therefore demyelination seems to be related to intrathecal IgM production.

After MP treatment mean (median) EDSS decreased from 4.4 (4.0) to 3.3 (3.0). Except for CSF/serum albumin (Q albumin) and IgM index, all CSF levels decreased significantly after MP. The mean CSF MBP returned to reference values. In the RR group the decrease in CSF MBP was significantly correlated with the change in EDSS ( $r=.39$ ). CSF MBP seems to be a good parameter for disease activity in relapsing MS. Following treatment CSF MBP was found to be related with the change in IgM index ( $r=.30$ ). MP treatment reduces CSF MBP and intrathecal IgM in a similar way indicating a relation between these 2 parameters.

## INTRODUCTION

Nowadays high dose intravenously administered methylprednisolone (MP) has an established role in the treatment of multiple sclerosis (MS) (1,2). In relapsing multiple sclerosis patients a significant decrease in clinical disability scores compared with controls (3), placebo (3,4) or other therapy regimens (5) is reported after MP treatment. A reduction is found in contrast enhancement of MS plaques on CT-scan (6) and recently also in gadolinium-DTPA enhancement on MRI (7), all suggesting that restoration of the Blood Brain Barrier (BBB) integrity is improved or accelerated after MP.

There have been relatively few reports discussing the effects of intravenously administered MP on cerebrospinal fluid (CSF) parameters in MS. A reduction in intrathecal IgG synthesis, myelin basic protein (MBP), anti-MBP, and anti- MAG (myelin associated glycoprotein) is reported (8-14). Qualitative IgG abnormalities, i.e. oligoclonal banding (OB) on iso-electric focusing (IEF) seems to be uninfluenced (3,8,9,15). How exactly the immune response and myelin breakdown in MS are tempered or influenced by MP treatment is unclear. We studied the effect of MP treatment on CSF MBP and immunoglobulin levels in 101 MS patients and the relation between CSF MBP and immunoglobulin levels.

## MATERIALS AND METHODS

A total of 101 consecutive MS patients ( 62 females ; 39 males) with clinically definite MS (CDMS) or laboratory supported definite MS (LSDMS), according to the criteria of Poser (16), who presented to the Department of Neurology of the University Hospital in Nijmegen with a symptomatic deterioration, were studied between 1988 and 1990. Mean age and disease duration (DD) was 35.4 (range 22-58) and 8.8 (range 0.7-24.8) years, respectively for the females; 35.2 (range 21-50) and 7.3 (range 0.3-23.0) years, respectively for the males.

All patients had a symptomatic deterioration and were classified to disease course: chronic progressive (CP) - 47 patients, relapsing remitting (RR)- 37 patients,

and a combination (CP+RR)- 17 patients. A relapse was defined as a symptomatic deterioration with the occurrence of one or more new symptoms or worsening of existing symptoms of less than 8 weeks but lasting longer than 24 hours without evidence of spontaneous improvement. A chronic progressive (CP) disease course was determined as a continuous symptomatic deterioration of neurological symptoms for at least 6 months. CP+RR was the combination of a chronic progressive disease course accompanied by relapses and remissions. All patients in the RR and CP+RR group had a relapse at the time at entrance into the study. No patient had used immunosuppressant agents 6 months or longer prior to entrance.

Reference values for CSF parameters were determined in a group of 35 patients (18 females, mean age 41.2, range 15-66 years; 17 males, mean age 45.0, range 17-65 years) with psychoneurosis without evidence of any organic, infectious or auto-immune inflammatory neurologic disease. The diagnoses were conversion disorders, tension headache and nervositas.

The patients were treated with 1000 mg MP daily for 10 consecutive days administered intravenously between 09.00 and 10.00 A.M. during approximately 15-20 minutes. No tapering dose was used. Therapy was tolerated well. Four major side-effects occurred : one epileptic seizure, one exacerbation of chronic bronchitis, one herpes zoster infection, and one peptic ulcer. Immediately before and after MP treatment blood samples and CSF taps were obtained simultaneously. Clinical scoring was performed according Kurtzke's Expanded Disability Status Scale (EDSS) (17) before and after MP treatment simultaneous with the blood and CSF examination by an examiner (S.T.F.M.F.) who was unaware of the CSF findings.

Peripheral venous blood and CSF samples were drawn according to standard procedures. Samples of blood and CSF were stored within 30 minutes at -70 C until assayed. IgG and albumin in unconcentrated CSF were measured by means of an automated kinetic turbidometric method described by Jongen et al (18). In brief sheep antihuman IgG (Boehringer, Mannheim, FRG) and rabbit antihuman albumin (Behringwerke AG, Marburg/Lahn, FRG) were used. Serum was always diluted (1:400) with saline, and both specimens (CSF and serum) were run in the same batch. IgM in CSF was measured by an ELISA method. Plates were coated with 150  $\mu$ l/well,

1/500 diluted rabbit antihuman IgM in PBS-buffer, pH 7.4 (DAKO, Copenhagen). CSF, 100 $\mu$ l and (1:10) diluted CSF and human IgM standards (Behringwerke AG, Marburg/Lahn, FRG) in duplicate were used per well. Rabbit peroxidase-conjugated antihuman IgM (DAKO, P125) was used for the enzyme reaction. The sensitivity level was 0.05 ng/ml.

We used the following ratios in analysis : Q Albumin = CSF albumin concentration \*  $10^5$  / Serum albumin concentration. Q IgG = CSF IgG concentration \*  $10^5$  / serum IgG concentration. IgG index = Q IgG / Q Albumin. CSF index for IgM was calculated with a similar equation. The calculation of intrathecal (de novo) IgG and IgM synthesis was performed according to the hyperbolic Reiber Formula (19).

For iso-electric focusing (IEF) CSF proteins were concentrated in micro-collodion Bag type under 1000 kPa nitrogen pressure. A constant amount protein of CSF and diluted serum were applied for IEF before and after MP treatment. For IEF paired CSF and serum examination was done with ready made LKB Ampholine PAG-Plates (product nr. 1804-101, pH range 3.5-9.5) and Coomassie blue staining. CSF oligoclonal banding was compared with serum. Myelin basic protein (MBP) was determined according to the instructions of the manufacturer (Diagnostic Systems Laboratories, Webster Texas USA, cat.nr. 1500), using a double antibody radioimmunoassay kit containing human basic protein (whole molecule) as antigen and rabbit anti-human MBP as anti-serum. Goat anti-rabbit gamma globulin was used as MBP precipitation reagent. Human MBP ( $J^{125}$  2  $\mu$ Ci/vial) was included in the test kit. The detection limit was 0.1  $\mu$ g/l ; the variation coefficient of the assay was 7.5%.

### *Statistical analysis*

Data before and after MP treatment were compared by means of Wilcoxon's signed rank test. The Kruskal Wallis test was used for differences between the three disease course groups. Relations between CSF data were calculated by means of Spearman's correlation coefficient. Hypotheses were tested two sided and p-values smaller than .05 were considered statistically significant. As data were not complete in all patients, the calculations were performed on numbers smaller than 101, but high enough to guarantee clear statistical decisions. To avoid crowding the numbers are not

mentioned in the tables.

## RESULTS

Table 1 shows the mean and median values of the clinical scoring (EDSS) and different CSF parameters before and after MP treatment for 101 MS patients. Before MP treatment, all mean CSF values, except for Q albumin, were significantly elevated compared to reference values. Oligoclonal CSF bands were present in 92% of the patients. Except for Q albumin, and IgM index, all CSF parameters decreased significantly after MP treatment. In 10 patients the oligoclonal banding pattern completely disappeared after treatment. The decrease in EDSS (1.0 point or more) was significantly ( $p<.05$ ) associated with young age ( $< 40$  years) and short disease duration ( $< 8$  years) and was not dependent of the disease course.

Table 2 gives the results for the three different disease course groups. Before treatment the mean CSF MBP levels were significantly ( $p=.03$ ) higher in the relapsing remitting (RR) and in the combined (CP+RR) group of MS patients compared to the CP MS group. CSF IgM ( $p=.014$ ) and intrathecal IgM synthesis ( $p=.06$ ) were higher in the relapsing remitting (RR) group versus the other 2 groups. After MP treatment only the mean CSF MBP levels for the three separate disease course groups of MS patients returned to normal values with respect to the reference values, while the mean values of all other CSF parameters investigated (except Q albumin) remained elevated.

Table 3 shows the relations between CSF parameters for the whole MS group ( $n=101$  patients). Before treatment CSF mononuclear cells were highly correlated with CSF MBP, IgG and IgM parameters. Moreover CSF MBP was significantly correlated with IgM parameters. IgM parameters were significantly correlated with IgG parameters.

le 1: Mean and median values of clinical scoring (EDSS) and CSF parameters in 101 MS patients before and after treatment with high doseavenous methylprednisolone (MP) (1000 mg daily for 10 consecutive days).

	Reference values		Unit	MS total (n=101 patients)				Sign		
				Pre MP		Post MP				
	Mean	P <sub>90</sub>			Mean	Median	> P <sub>90</sub>		Mean	Median
EDSS	-	-	-	4.4	4.0	-	3.3	3.0	-	****
Albumin •10 <sup>5</sup>	527	745	-	637	499	-	518	480	21.6%	ns
IF monon. cells	3	7	/3mm <sup>3</sup>	18	7	48.4%	9	6	43.8%	**
IF IgG	23.9	43.0	mg/l	62.5	46.0	59.4%	36.1	29.5	23.9%	****
G index	0.47	0.54	-	1.05	0.87	93.6%	0.98	0.74	83.9%	***
Intrath. IgG synthesis	0	0	mg/l	31.0	14.0	85.4%	11.6	4.0	70.1%	****
IF IgM	0.3	0.4	mg/l	2.1	0.6	65.6%	0.9	0.5	55.7%	***
M index	0.05	0.1	-	0.13	0.06	30.9%	0.12	0.06	31.0%	ns
Intrath. IgM synthesis	0	0	mg/l	1.0	0	32.3%	0.4	0	27.6%	**
F	0	0	-	5	6	92.1%	4	3	82.1%	****
IF MBP	0.6	1.2	µg/l	1.6	0.8	33.0%	0.7	0.5	8.0%	****

end: EDSS = Expanded Disability Status Scale; Q Albumin = CSF Alb/Serum Alb; CSF monon. cells = number of mononuclear cells; ath. Ig synthesis = Intrathecal Ig synthesis; IEF = iso-electric focusing, number of oligoclonal bands; CSF MBP = myelin basic protein; >P<sub>90</sub> = Percentage of MS patients with CSF value higher than P<sub>90</sub> of the reference value; Sign. = significance between pre and post MP treatment; ns = not significant; \*\* = p<.01; \*\*\* = p<.001; \*\*\*\* = p<.0001.



Table 2: Mean and median values of clinical scoring (EDSS) and CSF parameters in 101 MS patients before and after treatment with high doseavenous methylprednisolone (MP) (1000 mg daily for 10 days) given for the disease course.

	CP (n=47)				RR (n=37)				CP+RR (n=17)			
	Pre	Post	Mean (% > P <sub>90</sub> )	Median	Pre	Post	Mean (% > P <sub>90</sub> )	Median	Pre	Post	Mean (% > P <sub>90</sub> )	Median
EDSS	5.0	5.0	5.0	5.0	3.3	3.0	3.0	3.0	4.9	5.0	4.1	4.0
Albumin *10 <sup>5</sup>	601 (18.6%)	503	503 (19.5%)	455	751 (17.6%)	488	488	496	622 (29.4%)	593	545 (33.3%)	502
IF monon. cells	16 (39.5%)	6	7 (46.3%)	6	24 (51.4%)	9	9	6	18 (64.7%)	10	9 (40.0%)	7
IF IgG	65.4 (65.1%)	40.6	38.4 (23.8%)	28.0	71.5 (50.0%)	44.5	44.5	27.0	70.1 (64.7%)	59.0	36.5 (20.0%)	33.0
IF index	1.12 (93.0%)	0.90	1.17 (85.4%)	0.80	1.02 (91.2%)	0.84	0.84	0.66	1.09 (100%)	0.89	0.90 (86.7%)	0.77
path. IgG synthesis	31.4 (83.7%)	15.0	14.6 (73.2%)	5.0	29.2 (86.1%)	12.0	12.0	4.0	33.5 (88.2%)	18.0	12.1 (60.0%)	7.0
IF IgM	1.0 (53.5%)	0.5	0.7 (42.9%)	0.4	4.7 (72.2%)	0.7	0.7	0.6	1.8 (82.4%)	0.7	1.3 (60.0%)	0.5
IF index	0.08 (20.9%)	0.05	0.10 (24.4%)	0.06	0.23 (41.2%)	0.07	0.07	0.05	0.16 (35.3%)	0.07	0.14 (33.3%)	0.11
path. IgM synthesis	0.3 (20.9%)	0	0.1 (14.6%)	0	2.8 (44.4%)	0	0	0	1.1 (35.3%)	0	0.9 (53.3%)	0
F	6 (92.5%)	7	4 (84.2%)	3	5 (91.4%)	5	5	3	5 (92.9%)	5	4 (86.7%)	3
IF MBP	1.3 (20.9%)	0.6	0.7 (10.0%)	0.6	2.1 (44.1%)	1.1	1.1	0.5	2.3 (41.2%)	1.1	0.8 (6.7%)	0.5

Legend: Significance between pre and post treatment are mentioned; ns = not significant; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001; \*\*\*\* = p < 0.0001; CP = chronic progressive; RR = relapsing remitting; CP+RR = chronic progressive with relapses. % > P<sub>90</sub> = Percentage of MS patients with higher CSF value than P<sub>90</sub> reference value. The reference values are mentioned in Table 1.

Table 3: Spearman correlation coefficients of 6 CSF parameters in 101 MS patients before high dose intravenous methylprednisolone (MP) treatment.

	CSF Monon. cells	MBP	IgG index	Intrathecal IgG synthesis	IgM index	Intrathecal IgM synthesis
CSF Monon. cells	X					
MBP	.30 **	X				
IgG index	.36 ***	.06	X			
Intrathecal IgG synthesis	.37 ***	.13	XX	X		
IgM index	.28 **	.36 ***	.25 *	.29 **	X	
Intrathecal IgM synthesis	.30 **	.26 *	.25 *	.40 ****	XX	X

**Legend:** Monon. cells = CSF mononuclear cells; MBP = myelin basic protein; Significance: \* =  $p < .05$ ; \*\* =  $p < .01$ ; \*\*\* =  $p < .001$ ; \*\*\*\* =  $p < .0001$ . Without annotation means not significant.

Before MP treatment (not in table) EDSS of the total MS group was not significantly related with any of the CSF parameters. Only in the relapsing MS group there was a trend ( $p = .06$ ) towards a positive correlation between EDSS with CSF MBP ( $r = .34$ ). In the 2 other groups such a correlation could not be demonstrated. Following MP treatment (not in table) the decrease in the total CSF mononuclear cells was correlated with the decrease in CSF MBP level ( $r = .26^{*1}$ ), IgG index ( $r = .27^{*}$ ) and intrathecal IgG synthesis ( $r = .33^{**}$ ). The decrease in CSF MBP was significantly correlated with the decrease in IgM index ( $r = .30^{**}$ ); a trend ( $p < .10$ ) was found between the decrease in CSF MBP and lowering in EDSS following MP treatment ( $r = .18$ ). This correlation was statistically significant in the RR group ( $r = .39^{*}$ ) but not in the 2 other groups.

<sup>1</sup>  $p < .05$  \*,  $p < .01$  \*\*

## DISCUSSION

Multiple sclerosis is probably an auto-immune mediated inflammatory demyelinating disease from unknown origin. The pathophysiology of MS lesions may be divided in 4 different stages (20). Stage I stands for disruption of the blood-brain barrier (BBB) and is possible the first step in the demyelinating proces (21). Stage II stands for infiltration of lymphocytes and macrophages into the CNS; stage III for demyelination/myelin breakdown and stage IV for gliosis and scar formation ("sclerose en plaques"). Different lesions may be active at different stages simultaneously in the same patient (21).

Corticosteroids (high dose intravenously administered methylprednisolone) can improve BBB integrity (stage I) as found by CT scan (6) and gadolinium enhanced MRI (7) and have an immunosuppressive effect (3) on the inflammatory proces (stage II). The rationale for immunosuppressive treatment is that the amount of myelin breakdown/demyelination (stage III) can be tempered or possibly stopped in order to prevent evolution into stage IV.

In this study we have performed clinical scoring and CSF analysis in MS patients directly before and after MP treatment. This gives the opportunity to determine the effects of treatment on the different stages of the pathophysiological process of demyelination and to make relations between the clinical and CSF data.

### *Q Albumin*

In the present study the elevated Q albumin, which may represent a disturbed BBB function, showed no significant decrease after MP treatment. Others too have reported no statistical difference (13,22). This is not in accordance with radiological studies (contrast-enhanced CT and gadolinium-DTPA enhanced MRI) which demonstrated an improved BBB integrity after methylprednisolone (6,7). A possible explanation for this is that the molecular weight of albumin (60 kDa) and for example gadolinium-DTPA (550 Da) is quite different as might their routes of transport across the BBB be. Therefore both methods, which may represent BBB function, probably are not measuring the same aspect of the BBB.

### *CSF mononuclear cells*

In this study, after treatment, there was a significant drop in CSF mononuclear cells for the relapsing remitting (RR) and the combined (CP+RR) disease course group but not for the chronic progressive (CP) MS group. No correlation was found with the clinical scoring (EDSS). Baumhefner et al (11), Compston et al (10), and Trotter et al (15) did not find such changes, but their doses and total MP amounts (1000 mg MP intravenously for 3 days and 500 mg MP for 5 days) were much lower than our regimen. It is possible that the dose of 10 days 1000 mg MP may produce a stronger and significant reduction in CSF mononuclear cells, indicating a stronger effect on inflammatory activity than lower doses.

### *IgG parameters (CSF IgG, IgG index, intrathecal IgG synthesis)*

In the total MS group all three IgG parameters decreased significantly after MP treatment. IgG index decreased statistically significant only in the RR group. Decrease in intrathecal IgG synthesis after MP treatment is reported by several authors and is one of the most consistent findings about effects of MP on CSF parameters in both relapsing remitting and chronic progressive MS (3,8,9,11,15). In agreement with these studies we observed a significant reduction in intrathecal IgG synthesis in all 3 disease course groups of MS.

### *IgM parameters (CSF IgM, IgM index, intrathecal IgM synthesis)*

We found a trend towards a significant higher intrathecal IgM synthesis and significant higher CSF IgM in relapsing MS versus chronic progressive or the combined (CP+RR) disease course. Probably due to higher pre-treatment levels only in the relapsing MS group there was a significant decrease in CSF IgM, IgM index and intrathecal IgM synthesis after MP treatment. IgM parameters and EDSS were not significantly related to each other.

Recently several studies about IgM in MS have been published. Sharief and Thompson (23) detected intrathecal production of IgM (IgM oligoclonal bands in CSF and calculation of IgM index) in 55% of MS patients (n=150) which was found to correlate with disease activity defined as a recent relapse as well as the total number

of relapses. They concluded that intrathecal IgM synthesis as detected by CSF oligoclonal IgM bands is a useful parameter in monitoring disease activity in MS. Oligoclonal banding of IgM was found to be more specific for active disease than IgM index or the total CSF IgM amount. In an other study Sharief et al (24) established that CSF oligoclonal IgM is an indicator of recent immunological stimulation. We found a relation between IgM and disease activity, with a trend towards a higher intrathecal IgM synthesis and significantly higher CSF IgM in the relapsing MS group. Possibly the detection of CSF IgM abnormalities by an oligoclonal IgM method is more sensitive than the IgM index or calculated IgM synthesis.

### *Iso-electric focusing*

After MP administration we found a significant drop in the number of oligoclonal bands (OB) on IEF in the whole MS group depending most on the decrease in the CP and RR disease course group. In 10 patients oligoclonal banding disappeared completely; 5 patients had a RR disease course, 3 a CP course, and 2 a combined (CP+RR) disease course. No correlation was found with the EDSS. This disappearance of oligoclonal banding pattern is reported in a small number of MP treated MS patients (3,15). Baumhefner et al (11) observed no significant changes. For determination of the IEF before and after MP treatment we have applied a standardized amount of protein and not a constant IgG amount in concentrated CSF. Because we found significantly lower CSF IgG levels after MP treatment the possibility occurs that faint bands before treatment are invisible after treatment. Anyhow in our opinion the significant difference of the number of oligoclonal bands between pre and post treatment can not be completely elucidated by the used method. In accordance with Tourtellotte et al (9), Durelli et al (3) sustained the hypothesis of a steroid-resistant colony of IgG synthesizing immunocytes within the CNS (clones of B-cells) because of the resistance of oligoclonal banding (OB). Our data suggest that probably the disease course, the dose and route of MP administration are important for the effects of MP on the decrease and disappearance of oligoclonal banding pattern after MP treatment.

### *CSF myelin basic protein*

CSF MBP levels were significantly different in the 3 disease course groups of MS. In the RR and the combined (CP+RR) MS group the levels were higher than in the CP group. In the relapsing MS group before treatment we found a positive trend between CSF MBP and clinical scoring (EDSS) and a significant correlation between their changes following MP treatment. Elevated levels of CSF MBP have been found in MS patients with active disease and seem to correspond with disease activity (25-29). In a study of Martin-Mondière et al (30) MBP was not detected in any MS patient with inactive or slowly progressive disease nor in any patient during exacerbations with only recurrence of old signs or symptoms. Thompson et al (31) found a significant correlation between CSF MBP and relapse severity in MS patients seen within 4 weeks of the onset of symptoms. Recently Barkhof et al (22) found a significant correlation between CSF MBP, EDSS, and the number of gadolinium enhancing lesions by MRI in relapsing MS patients. Using gadolinium-enhanced MRI Thompson et al (32) found significantly more enhancing lesions in the group of secondary progressive (compare CP+RR) MS than in the primary progressive group (CP) which may indicate a difference in inflammatory disease activity. These two studies demonstrate that both parameters, CSF MBP and the number of enhancing lesions by gadolinium-enhanced MRI, seem to correspond with disease activity in MS.

In our study a significant drop in CSF MBP levels after MP treatment was demonstrated. Warren et al (12,13,27) found in exacerbating MS patients a significant drop in CSF MBP after MP treatment (160 mg/day or 2 g/day for 10 days) in contrast to ACTH treatment (60 units/day intravenously for 10 days). MS patients with progressive disease showed relatively insignificant changes in MBP. Our results confirm those findings with significant decreases in the RR and CP+RR MS group and not in the CP group. All these results from different studies indicate that CSF MBP might be a good indicator of disease activity especially in relapsing MS patients.

### *Correlations between CSF parameters before and following MP treatment*

Before and after MP treatment CSF mononuclear cells were significantly correlated with MBP, IgG index and intrathecal IgG synthesis. There was no

significant correlation between MBP and IgG parameters. This is in accordance with findings of Warren and Catz (27) and Lamers et al (33) who also found no correlation between CSF MBP and CSF IgG measurements suggesting that demyelination and local IgG activity are not related. The only significant relationship before and after MP treatment between MBP and immunoglobulines was MBP and IgM index, indicating first that demyelination (stage III) occurs at the time of IgM production (stage II) and second that the effect of MP treatment is accompanied by a correlative decrease of myelin breakdown (expressed by decrease of MBP) and decrease of IgM production. This suggests that MP actually interferes with the inflammatory process of demyelination. After MP treatment we found a significant reduction in CSF mononuclear cells (stage II). This may implicate a reduction in the number of B-lymphocytes producing immunoglobulines in the CNS compartment.

In summary CSF MBP may be a good parameter for disease activity and also in evaluation of therapy, especially in MS patients with relapsing disease course. CSF MBP seems to be related with intrathecal IgM production and not with IgG synthesis in the CNS. Methylprednisolone treatment reduces both parameters in a correlative way. The CSF MBP levels return to reference values, however IgM production does not normalize. This means that the immunological process of myelin breakdown is not completely eradicated and may continue.

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## **CHAPTER 5, section 2**

### **T-CELL SUBSETS IN THE CEREBROSPINAL FLUID AND PERIPHERAL BLOOD OF MULTIPLE SCLEROSIS PATIENTS TREATED WITH HIGH-DOSE INTRAVENOUS METHYLPREDNISOLONE**

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## SUMMARY

To determine the effects of high-dose intravenous methylprednisolone (MP) on lymphocytes and lymphocyte subpopulations in the cerebrospinal fluid (CSF) and peripheral blood (PB) in multiple sclerosis (MS) patients, we studied 67 patients with definite MS treated with MP. They were classified according to the disease course: 32 chronic progressive (CP) patients, 25 relapsing-remitting (RR) patients, and 10 patients with a chronic progressive disease course accompanied by relapses and remissions (CP+RR). MS patients were treated with 1000 mgr intravenous MP daily for 10 consecutive days. Before and after MP treatment we simultaneously studied CSF and PB CD3+, CD4+, CD8+, CD20+, and Ia1+ cell subsets. Kurtzke's Expanded Disability Status Scale (EDSS) was used for clinical evaluation. Progression rate was defined as the ratio of EDSS to disease duration. Thirteen patients with lumbar disk herniation were investigated as controls. Before MP, we found in MS patients, especially in the CP group, significantly lower CD4+ T-cell percentages in the PB with respect to controls ( $p < 0.05$ ). The percentage of CD4+ T-cells in the CSF of MS patients was significantly higher compared with PB ( $p = 0.0001$ ), and tended to be higher than in controls ( $p = 0.072$ ). The CSF mononuclear cell counts were significantly correlated with higher percentages of CSF CD3+ ( $r = 0.40$ ) and CD4+ ( $r = 0.47$ ) T-cells and lower CSF CD8+ ( $r = -0.33$ ) T-cell percentages. B-cell percentages in the CSF were significantly elevated compared with controls for all MS groups. No relation could be obtained between T- or B-cell subsets and EDSS or progression rate. After MP, a significant decrease in PB CD8+ T-cell percentage and simultaneously an increase of the percentage CD8+ T-cells in CSF was noted in the entire MS group and in the CP and RR MS patients. Except for the CP+RR MS patients, CD4+ T-cell percentages in the PB or CSF showed insignificant changes. Our findings support the view that in MS MP might affect the inflammatory process of demyelination by a selective and dissociative effect on T-suppressor/cytotoxic cells in the PB and CSF.

## INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system of unknown origin. Contradicting findings have been reported about T-cell subsets in the peripheral blood (PB) and cerebrospinal fluid (CSF) of active MS patients. Decreased CD8+ (suppressor/cytotoxic) T-cells in PB and CSF with an increase in the ratio CD4+/CD8+ which correlates with an increase in T-cell activation are found to be related to increased disease activity [1-6]. However, these findings could not be confirmed in other studies [7-9].

High dose intravenous methylprednisolone (MP) often results in a rapid clinical improvement in patients with exacerbating MS [10,11] and as well as a reduction of inflammation and demyelination expressed by a decrease of intrathecal IgG synthesis and CSF myelin basic protein [12,13]. After MP, suppression of gadolinium enhancement on magnetic resonance imaging (MRI) of the brain is reported indicating restoration of the disturbed blood brain barrier (BBB), which correlates well with clinical improvement [14,15].

Whether MP interferes primarily with the process of demyelination or reacts non-specifically with its mediators is not clear. MP might act by interference with cellular immune mechanisms. Therefore, we studied the effects of MP on lymphocytes and lymphocyte subpopulations in CSF and PB of MS patients. In order to determine how the changes (after MP) of T-cell subsets in PB are related to the changes in CSF, T-cell subsets were simultaneously studied in both compartments.

## PATIENTS AND METHODS

Between 1988 and 1990 67 consecutive patients (42 women, 25 men,) with clinically definite (CD) or laboratory supported definite (LSD) MS [16] were studied at the University Hospital Nijmegen. The mean age was 35.5 years (range 21-54 yrs.), and mean disease duration was 8.5 years (range 0.5-24 yrs.). All patients suffered a clinical deterioration and were classified according to disease course: chronic progressive (CP- 32 patients), relapsing-remitting (RR- 25 patients), and chronic progressive

with relapses and remissions (CP+RR- 10 patients). A relapse was defined as either the onset of new symptoms and signs or a deterioration in existing symptoms and signs within the previous 8 weeks and lasting longer than 24 hours, without evidence of spontaneous improvement. CP was defined as a continuous clinical deterioration of neurological symptoms and signs for at least 6 months. All patients in the RR and CP+RR disease course groups had a relapse at the moment of entrance into the study. No patient had used immunosuppressant agents 6 months prior to entrance into the study.

Patients were treated with 1000 mg methylprednisolone-acetate (MP) daily for 10 consecutive days administered intravenously between 9:00 and 10:00 a.m. over approximately 15-20 minutes. No tapering dose was used after the 10 day MP course.

Before and after MP treatment, the clinical status was scored according to Kurtzke's Expanded Disability Status Scale (EDSS) [17] by a neurologist (SF) who was unaware of the CSF and PB findings. Progression rate was defined as the ratio of EDSS to disease duration. Thirteen patients (8 women, 5 men), aged 30-69 years (mean 42.1 yrs.), with herniated lumbosacral discs served as controls.

Immediately before and after MP treatment, PB and CSF samples were obtained simultaneously according to standard procedures. White blood cell count and leukocyte differentiation were performed routinely using a Coulter Counter and May-Grunwald Giemsa staining. For determination of lymphocyte subpopulations, lymphocytes from heparinized PB were isolated by Ficol-isopaque density gradient centrifugation ( $1.085 \text{ g/cm}^3$ ). The remaining erythrocytes were lysed and the cells were washed twice and labelled according to the procedure described by Reinherz et al. [18]. The following monoclonal antibodies (MAB) were used: anti-CD3 (OKT3, total T cells), anti-CD4 (OKT4, helper/inducer T-cells), anti-CD8 (OKT8, suppressor/cytotoxic T-cells), anti-OKIa1 (activated cells, Ortho Pharmaceutical, Raritan, N.J.), and anti-CD20 (B-cells, Coulter clone, Luton, Beds; England).

For CSF cell analysis we used a micromethod. The CSF was centrifuged (10 minutes,  $400 \times g$ ,  $20^\circ\text{C}$ ) and the supernatant was removed. The remaining cell suspension was aliquoted over 6 Eppendorf cups ( $\pm 50 \mu\text{l}$  each). The cells were washed once with 2% bovine serum albumin in Tris buffered minimal essential

medium. The supernatant was removed, and to the remaining cell suspension ( $\pm 50 \mu\text{l}$ )  $2 \mu\text{l}$  MAB was added and the suspension was incubated for 30 min at  $4^\circ\text{C}$ . After two wash steps, the suspensions were incubated with  $25 \mu\text{l}$  goat anti-mouse fluoresce isothiocyanate conjugate (GAM/FITC). After incubation, the cells were washed twice and the cell pellets were fixed overnight in  $100 \mu\text{l}$  1% paraformaldehyde. Then  $10 \mu\text{l}$  ethidium bromide ( $10 \text{ g/ml}$  PBS) was added for nuclear staining. Subsequently, the cell suspensions were cytocentrifuged and embedded in glycerol/ $\text{NaN}_3$  ( $25\text{mg}$   $\text{NaN}_3$  and  $10\text{mg}$  paraphenylene diamine in  $1 \text{ ml}$  aqua bidest and  $9 \text{ ml}$  glycerol). For cell counting we employed a Zeiss fluorescence microscope equipped with a HBO 50W mercury lamp and the filter combination BP 485/20, FT 510, LP 520, using an oil immersion objective  $63 \times 1.30$  and a pair of 8X oculars. At least 100 cells in duplicate were counted per monoclonal antibody. In both PB and CSF the proportion of lymphocytes was expressed as the percentage of the total mononuclear cell count.

## Statistics

Differences between the three disease course groups were tested by means of the Kruskal-Wallis test. Data before and after MP treatment were compared by means of the Wilcoxon's signed rank test. Relations between PB and CSF data were calculated by means of Spearman's correlation coefficient. P-values of 0.05 or less (two-sided) were considered as statistically significant; p-values between 0.05 and 0.10 were considered as trends.

## RESULTS

### *T-cell subsets in PB and CSF of 67 MS patients before and after MP treatment (Table 1)*

Before MP treatment, CSF B-cells were significantly higher ( $p < 0.005$ ), PB  $\text{CD4}^+$  T-cells and PB  $\text{CD4}^+/\text{CD8}^+$  ratio levels were significantly lower ( $p < 0.05$ ) in MS patients than in controls. The percentage of CSF  $\text{CD4}^+$  T-cells in MS patients tended to be higher compared with controls ( $p = 0.072$ ) (Figure 1). In the MS patients, the percentage of  $\text{CD4}^+$  T-cells in the CSF was significantly higher than in

the PB ( $p = 0.0001$ ) (not in the table). After MP treatment, a significant increase was found in the CSF CD8+ cells ( $p = 0.0009$ ) resulting in a decrease of the CSF CD4+/CD8+ ratio, and a decrease in PB CD8+ T-cells ( $p = 0.0006$ ) resulting in an increase of the PB CD4+/CD8+ ratio (Figure 2). Spearman's correlation coefficients between the PB and CSF variables before MP treatment are shown in Table 2. There is a clear positive correlation between each corresponding T-cell subset percentage in the CSF and PB. Table 3 shows the correlation coefficients between the total number of CSF mononuclear cells and T-cell subsets in the PB and CSF. A high cell count of CSF mononuclear cells correlates with higher percentages of CSF CD3+ and CD4+ T-cells and lower CSF CD8+ T-cell percentages, and subsequently higher CSF CD4+/CD8+ ratios.

Table 1: EDSS, peripheral blood (PB) and cerebrospinal fluid (CSF) T subsets in 67 Multiple Sclerosis (MS) patients before and after treatment with high-dose intravenous methylprednisolone (MP) and in 13 controls with lumbosacral disk herniation.

	Controls	Unit	MS patients		p-value
			before MP	after MP	
EDSS	-	-	4.3	3.4	0.001
PB leukocytes	7.2 (2.8)	$\times 10^9/L$	6.3 (2.3)	10.4 (4.5)**	0.0001
lymphocytes	29.9 (5.0)	%	27.5 (8.8)	19.1 (10.3)***	0.0001
CD3+	66.6 (7.3)	%	61.3 (11.8)	59.9 (11.0)*	ns
CD4+	46.0 (8.9)	%	37.3 (12.7)*	37.1 (13.6)*	ns
CD8+	20.3 (4.5)	%	23.8 (9.1)	21.0 (7.4)	0.0006
CD4+/CD8+	2.4 (0.7)	-	1.9 (1.1)*	2.1 (1.3)	ns
Ia1+	8.8 (4.4)	%	9.1 (5.8)	10.7 (6.2)	ns
B1	5.5 (2.9)	%	5.2 (4.1)	5.9 (3.6)	ns
CSF mononuclear cells	2.8 (2.4)	$/3mm^3$	19.6 (33.7)***	11.1 (15.6)***	0.0005
CD3+	76.8 (6.1)	%	79.2 (11.2)	78.7 (10.4)	ns
CD4+	49.8 (9.7)	%	55.7 (13.7)#	51.6 (11.7)	ns
CD8+	18.8 (5.9)	%	21.9 (7.6)	25.3 (9.0)*	0.0009
CD4/CD8+	2.7 (1.1)	-	2.9 (1.4)	2.3 (1.0)	0.0001
Ia1+	7.3 (2.2)	%	9.1 (5.5)	12.8 (8.1)*	0.0075
B1	2.4 (3.2)	%	5.6 (4.3)**	7.8 (5.8)***	ns

Legend: Mean levels with one standard deviation are reported. Wilcoxon's rank sum test;

# =  $p < 0.10$ ; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  tested against the controls (second column); without annotation corresponds to values which are not significantly different with respect to the controls. In the last column the p-values indicate the significance between the levels before and after treatment. EDSS= Expanded Disability Status Scale; ns= not significant. The percentages of Ia1+ and B1 cells refer to the percentages of mononuclear cells in the PB and CSF, respectively.

## CD4+ T-cells

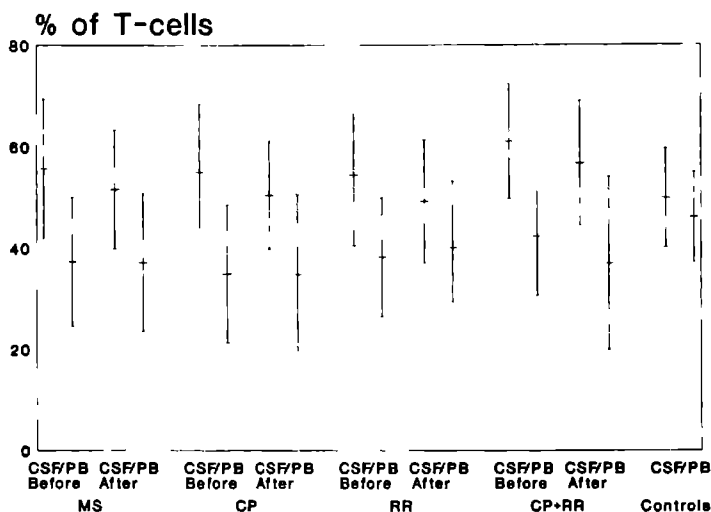


Figure 1. Mean percentages ( $\pm 1$  standard deviation) of CD4+ T-cells in the CSF and PB of 67 MS patients before and after high-dose intravenous MP, according to the course of the disease, and in 13 controls. CP= chronic progressive; RR= relapsing-remitting; CP+RR= combination.

## CD8+ T-cells

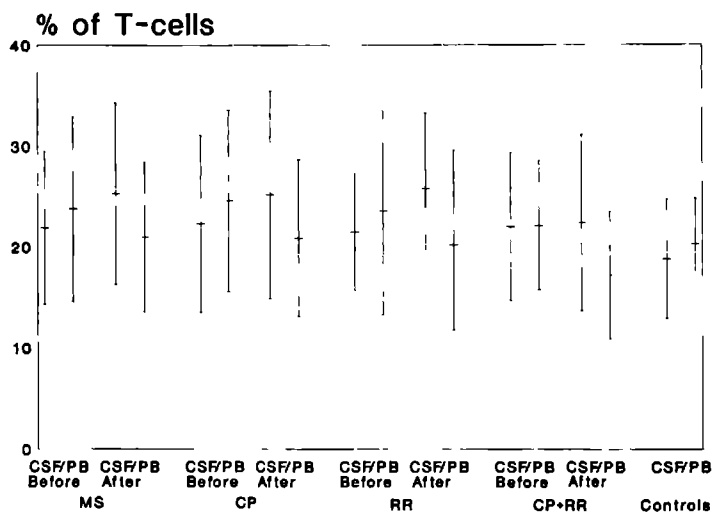


Figure 2. Mean percentages ( $\pm 1$  standard deviation) of CD8+ T-cells in the CSF and PB of 67 MS patients before and after high-dose intravenous MP, according to the course of the disease, and in 13 controls.



Table 2: Spearman correlation coefficients between the percentages of T-cell subsets in the PB and CSF of 67 MS patients before MP treatment.

	CSF CD3+	CD4+	CD8+	CD4+/CD8+	Ia1+	B1
<b>PB</b>						
CD3+	<b>0.34**</b>	0.28*	ns	ns	ns	ns
CD4+	<b>0.36**</b>	<b>0.30*</b>	ns	ns	ns	ns
CD8+	ns	ns	<b>0.34**</b>	ns	ns	ns
CD4+/CD8+	ns	0.24*	-0.32*	<b>0.29*</b>	ns	ns
Ia1+	ns	ns	ns	ns	<b>0.36**</b>	<b>0.28*</b>
B1	ns	ns	ns	ns	<b>0.50***</b>	<b>0.45***</b>

Legend: ns = not significant; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ .

Table 3: Spearman correlation coefficients between the CSF total number of mononuclear cells with the percentages of T-cell subsets in the PB and CSF of 67 MS patients before MP treatment.

CSF Mononuclear cells	CD3+	CD4+	CD8+	CD4+/CD8+	Ia1+	B1
<b>PB</b>	ns	0.25*	ns	0.25*	ns	ns
<b>CSF</b>	0.40***	0.47****	-0.33**	0.46****	ns	ns

Legend: See Table 2.

### *Relation between clinical variables and T-cell subsets in PB and CSF before and after MP treatment*

The EDSS-score decreased significantly after MP for the entire MS group and in all subgroups (Tables 1 and 4). The CSF mononuclear cell counts were significantly higher in the patients with a RR or CP+RR disease course than in the patients with a CP course ( $p < 0.05$ ). The CSF mononuclear cell counts were negatively correlated with age ( $r = -0.44$ ;  $p < 0.001$ ) and disease duration ( $r = -0.32$ ;  $p < 0.01$ ). None of the other cellular variables were significantly correlated with the course or duration of the disease, age, EDSS, or rate of progression of the disease. The values of the different PB and CSF variables for the various courses of disease are shown in Table 4. The changes of the T-cell subset percentages after MP were independent of the changes in the EDSS and not significantly correlated with the various courses and rate of progression of the disease (not in the table).

le 4: EDSS, PB and CSF T-cell subsets in 67 MS patients with various courses of disease, before and after treatment with high dose intravenous MP.

	Chronic Progressive (CP) (N=32)			Relapsing-Remitting (RR) (N=25)			Chronic Progressive with Relapses/Remissions (CP+RR) (N=10)		
	Before	After	P-value	Before	After	P-value	Before	After	P-value
EDSS	5.0	4.2	0.0001	3.0	1.9	0.001	5.2	4.6	0.05
T leukocytes	6.9 (2.8)	11.5 (6.0)*	0.000001	6.8 (2.5)	10.3 (3.6)**	0.0001	5.6 (1.1)	10.7 (3.9)*	0.016
lymphocytes	25.8 (9.7)	17.3 (10.8)	0.000001	27.0 (9.2)	21.3 (10.7)**	0.0156	31.0 (6.9)	21.7 (6.8)**	0.023
CD3+	59.8 (11.9)*	58.9 (14.1)*	ns	62.0 (13.4)	59.1 (12.9)	ns	63.9 (6.5)	57.1 (18.8)	ns
CD4+	34.9 (13.6)**	34.8 (15.8)**	ns	38.2 (11.7)	40.0 (13.2)	ns	42.3 (11.7)	36.9 (17.1)	0.05
CD8+	24.6 (9.0)	20.9 (7.8)	0.01	23.6 (10.3)	20.2 (9.4)	0.04	22.1 (6.4)	17.2 (6.3)	ns
CD4+/CD8+	1.7 (1.2)*	1.9 (1.3)	ns	1.9 (1.0)	2.6 (1.9)	ns	2.2 (1.1)	2.5 (1.3)	ns
IL+	9.6 (5.6)	10.2 (6.3)	ns	8.0 (5.5)	9.4 (5.3)	ns	10.1 (6.9)	11.3 (8.2)	ns
IF mono-	6.3 (4.8)	6.5 (4.7)	ns	3.8 (2.9)*	6.1 (3.9)	0.05	5.1 (3.7)	4.6 (2.6)	ns
clear cells	8.2 (6.9)**	7.2 (5.9)	ns	34.5 (48.7)***	16.8 (21.6)***	0.008	14.4 (11.5)**	9.2 (8.9)*	ns (0.086) to be continued
CD3+	78.9 (10.5)	76.3 (9.8)	ns	78.0 (12.7)	78.3 (10.7)	ns	83.0 (9.9)	84.4 (9.8)*	ns
CD4+	55.0 (14.3)	50.4 (10.6)	ns	54.4 (13.9)	49.2 (12.2)	ns	61.0 (11.3)	56.7 (12.3)	ns
CD8+	22.3 (8.8)	25.2 (10.3)*	0.03	21.5 (6.1)	25.8 (7.5)**	0.03	22.0 (7.3)	22.4 (8.7)	ns
CD4+/CD8+	3.0 (1.7)	2.4 (1.2)	0.005	2.8 (1.1)	2.0 (0.8)	0.003	3.0 (1.1)	3.0 (1.5)	ns
IL+	10.2 (6.2)	13.6 (9.4)*	ns	7.4 (3.9)	11.5 (6.4)*	0.02	9.9 (5.9)	11.3 (6.5)*	ns
	6.4 (4.2)	7.0 (5.9)	ns	4.3 (3.4)*	7.1 (5.4)	0.03	5.9 (5.9)	9.6 (7.0)	ns

end: Mean levels with one standard deviation are reported. Wilcoxon's rank sum test; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  tested against the controls (second column); without annotation corresponds to values which are not significantly different with respect to the controls. p-values in the columns indicate the significance between the levels before and after treatment for the various courses of disease. ns = not significant. The percentages of Ia1+ and B1 cells refer to the percentages of mononuclear cells in the PB and CSF, respectively.

## DISCUSSION

The most striking finding in this study is the different effect of MP on CD8+ T-cells in PB and CSF. The percentage of CD8+ T-cells (with corresponding changes in the CD4+/CD8+ ratios) decreases in the PB and increases in the CSF concomitantly, independent of the course of disease. The decrease of CD8+ T-cells in the PB and increase in the CSF may represent migration of these CD8+ T-cells into the central nervous system [19]. Although most cells in the CSF are of hematogenous origin [20], both selective migration of cells through the blood brain barrier and intrathecal expansion could contribute to different T subsets in PB and in CSF [21,22]. Weiner et al. [23] proposed that (chronic) active lesions in the CNS of MS patients could be the sequestration site of the CD8+ T-cells. Hayashi et al. [24] and McCallum et al. [25] found also a predominance of CD8+ lymphocytes in the perivascular and parenchymal compartments of MS lesions. Raine [26] stated that the strongest evidence for cessation or reversal of the immunological events in the resolving MS lesion lies in finding fewer CD4+ T-cells in the parenchyma and an increased presence of CD8+ T-lymphocytes, particularly around blood vessels. Estes et al. [27] indicated that both CD4+ and CD8+ lymphocytes are present in active MS lesions.

Data on the effects of corticosteroids on T-cell subpopulation in MS patients are often conflicting. Compston et al. [28] reported no statistically significant effects on OKT4 and OKT8 cells in the PB of MS patients after MP treatment. Salmaggi et al. [29] found increased numbers of CD3+ and CD4+ T-cells, with an increased CD4+/CD8+ ratio, in the PB of MS patients during dexamethasone treatment followed by a decrease 1 and 2 weeks after the end of the treatment. Kirk and Compston [30] reported no alterations in CD4+ and CD8+ T-cells, or activated lymphocytes in the PB in 25 MS patients who were treated with 0.5 g methylprednisolone intravenously daily for 5 days. CSF analysis was not performed in these three studies.

In the present study we found a significantly lower percentage of CD4+ T-cells in the PB of MS patients, especially in the CP group. In MS patients, the percentage of CD4+ T-cells in the CSF was significantly higher than in the PB and the CSF

CD4+ T-cell percentage tended to be higher than that of controls. These findings are in accordance with the study of Scolozzi et al. [31]. In our study, normal percentages for CD8+ T-cells in the PB and CSF of the MS patients, independent of the course of the disease, were obtained. Other studies have revealed a loss of circulating CD8+ (suppressor/cytotoxic) T-cells in PB or CSF in active or chronic progressive MS with subsequently higher CD4+/CD8+ ratios [6,18,32-36]. However, unchanged levels of CD8+ T-cells have been reported by others, both in PB or CSF [8,9]. One reason for the conflicting results may be that different methods (staining by fluorescent or enzyme-linked antibodies, type of antibody used, enumeration of the cells by microscopical evaluation or by flow cytometry) are used for determine the PB and CSF cells, usually requiring high CSF cell counts. We used a micro fluorescence method with the advantage of performing lymphocyte-subset analysis in the CSF in the absence of pleocytosis.

Comparing the different disease courses in MS, we found a significant relationship between disease course and CSF pleocytosis: MS patients in acute relapse (RR or CP+RR) had higher CSF cell counts than patients with chronic progressive (CP) disease. This is consistent with previous reports [31,37,38]. No relationship could be found between the percentages of T-cell subsets in CSF or PB with the different courses of disease or other clinical variables (EDSS, disease duration, progression rate).

The CSF pleocytosis in the MS patients in our study consisted predominantly of CD3+ T-cells, whose percentage is significantly higher in CSF than in PB. This is in agreement with previous studies [39,40]. Furthermore, the CSF pleocytosis is positively correlated with the percentage CSF CD4+ T-cells and negatively correlated with the percentage CSF CD8+ T-cells. A correlation of higher CSF mononuclear cell counts with a decreased percentage of suppressor/cytotoxic T-cells in the CSF has been found also by others [2,41]. Vanderbark et al. [22] stated that a selective increase of CD4+ T-lymphocytes account for increased cellularity in the CSF.

The percentage of B-lymphocytes in the CSF of MS patients was significantly higher than in controls; this was not found in PB. Prior studies, using different methodologies, have found either higher or lower percentages of B-cells [42,43]. The

Ia1 antigen, usually present on monocytes, B-lymphocytes, and some activated CD4+ T-cells [18], was found to be normal in PB and CSF. Similarly, Hauser et al. [2] have not found an increased percentages of Ia1+ cells in the PB and CSF of MS patients.

After treatment with intravenous 1000 mg MP daily for consecutive 10 days, we found a leukocytosis in the PB, a decrease in the lymphocyte percentage, and unchanged PB CD3+ and CD4+ T-cell percentages. The reduction of the percentage of lymphocytes in the PB after MP is probably due to a redistribution of lymphocytes from intravascular into peripheral compartments rather than due to killing of cells [44,45,46].

In conclusion, our findings suggest that in MS patients MP increases the T suppressor/cytotoxic subset in the CSF, possibly through migration of CD8+ T-cells from the PB compartment into the CNS. So, MP might have a selective and dissociative effect on CD8+ T-cells in the PB and CSF in MS and MP might affect the inflammatory process of demyelination by interference with T-suppressor/cytotoxic cells.

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## **CHAPTER 5, section 3**

### **DIFFUSE CENTRAL NERVOUS SYSTEM INVOLVEMENT IN SYSTEMIC LUPUS ERYTHEMATOSUS: INTRATHECAL SYNTHESIS OF THE FOURTH COMPONENT OF COMPLEMENT**

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## SUMMARY

In extracerebral systemic lupus erythematosus (SLE), the complement system plays a prominent pathogenic role and decreased serum concentration of the fourth component (C4) is a reliable indicator of systemic disease activity. In diffuse CNS-SLE, however, the pathogenic role of complement is less clear. In 12 patients with active diffuse CNS-SLE, presenting with delirium (4), organic personality syndrome (3), or generalized seizures (5), we determined the cerebrospinal fluid indices (CSF indices) of the complement components C3, C4, and factor B, and of the immunoglobulins IgG, IgA, and IgM. There was a significant increase of the C4 index in these patients compared to controls, and a significantly higher CSF C4 index in patients with an increased IgM index. We conclude that intrathecal C4 is being produced in diffuse CNS-SLE.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the production of autoantibodies against a large number of antigens, as well as the deposition of immune complexes of antigen and antibody (IC), and complement components in various organs <sup>1</sup>. Central nervous system involvement in SLE (CNS-SLE) is a common and serious manifestation, occurring in approximately half of all patients at some time during the course of their illness <sup>2,3</sup>. The most common neuropsychiatric manifestations of SLE - delirium, organic personality syndrome, cognitive impairment, and generalized seizures - reflect diffuse brain dysfunction <sup>3</sup>. Presumably, they result from the combined effect of antineuronal antibody binding to brain tissue and immune complex-mediated vasculopathy <sup>3,4</sup>, although the exact pathogenic mechanisms have not yet been elucidated.

In patients with active CNS-SLE there are increased CSF indices of IgG, IgA, and especially IgM, indicating intrathecal immunoglobulin synthesis <sup>5,6</sup>. There are also IC in the CSF of CNS-SLE patients <sup>7</sup>. Since IC-induced activation of the classical complement system plays a prominent role in SLE pathogenesis <sup>8</sup>, intrathecal complement activation and consumption might occur in CNS-SLE <sup>9</sup>. If so, increased complement synthesis is to be expected, analogous to complement production after hypocomplementemia in extracerebral SLE <sup>10,11</sup>. In fact, in CNS-SLE patients, CSF concentrations of the fourth component of complement (C4) are abnormally low <sup>9</sup> or normal <sup>12,13</sup>. However, for several reasons, measurement of only CSF concentrations is not an appropriate method to detect complement involvement in CNS-SLE. Firstly, in view of the wide normal ranges of CSF complement concentrations, minor changes do not necessarily lead to abnormal levels. Secondly, CSF complement concentrations are derived from, and related to, serum concentrations; therefore abnormally low CSF concentrations do not necessarily reflect complement consumption within the CNS. Thirdly, impairment of the blood-brain barrier (BBB) results in transudation of complement components and an increase of CSF concentrations. Calculation of the CSF complement indices, analogous to CSF immunoglobulin indices <sup>14</sup>, meets these objections and might demonstrate intrathecal synthesis or consumption of

complement.

We determined the values of the 3rd and the 4th complement components (C3, C4) and factor B, as well as IgG, IgA, IgM, and albumin, in paired serum and CSF samples from 12 patients with diffuse CNS-SLE and examined the respective CSF indices.

## PATIENTS

We studied 12 patients with diffuse CNS-SLE, who had previously or concurrently satisfied the 1982 American Rheumatism Association (ARA) diagnostic criteria for definite SLE <sup>15</sup>. Nine were woman, 3 men. Ages ranged between 17 and 61 years (Table 1). They were seen in the period 1983 to 1988 when hospitalized at the University Hospital Nijmegen. Clinical assessment included internal, neurologic, and psychiatric examination. All patients showed unequivocal psychiatric or neurologic signs and symptoms of progressive diffuse CNS dysfunction, suggesting active CNS-SLE. Psychiatric diagnoses according to the Revised 3rd edition of the Diagnostic and Statistical Manual of Psychiatric Disorders (DSM-III-R) <sup>16</sup> were delirium with cognitive impairment (2), delirium without cognitive impairment (2), organic personality syndrome (OPS) with cognitive impairment (1), and OPS without cognitive impairment (2). Generalized seizures (5), in one patient associated with cognitive impairment, accounted for the neurologic diagnoses (Table 1). All patients showed abnormalities on routine CSF studies (total protein concentration, total leucocyte count), electro-encephalogram (generalized slowing of background activity 6, generalized spikes 4), or cerebral computerized tomography (slight cortical marking 2; slight cortical and central marking 5, moderate cortical and central marking 1, and severe cortical and central marking 1). At the time of diagnosis, CNS dysfunction could not be attributed to infectious disease, metabolic disturbances, or psychogenic reactions. Steroid-induced delirium was unlikely, since all delirium patients improved after increases of steroid dosage. In patients 2 and 7 (Table 1) a concomitant SLE nephritis existed, but serum urea concentrations did not suggest uremic encephalopathy. In no patient did the diagnosis have to be changed during the course

of the disease.

The control group consisted of 25 patients without evidence of systemic connective tissue disease, and infectious or autoimmune inflammatory neurologic disease. Diagnoses included radiating low back pain, without abnormalities on subsequent myelography (21), and psychogenic conversion (4). CSF total protein concentration and cell count were within normal limits, and erythrocyte concentration was less than  $50/\text{mm}^3$ , excluding a traumatic puncture.

After informed consent blood and CSF were obtained for diagnostic purposes.

## METHODS

In CNS-SLE patients and in controls, peripheral venous blood and CSF were drawn simultaneously according to standard procedures. Samples of serum and CSF were stored within 30 minutes at  $-70^\circ\text{C}$  until assayed. In 1:100 diluted serum and unconcentrated CSF specimens the concentrations of C3, C4, and factor B were measured nephelometrically, using antisera purchased from Kallestad Laboratories Inc. (Austin, Texas, USA). In each patient CSF and serum samples were measured in the same batch.

IgG, IgA, and albumin in unconcentrated CSF of the CNS-SLE patients were measured by means of an automated kinetic turbidimetric method. The reaction time was approximately two minutes. Sheep antihuman IgG and IgA (Boehringer, Mannheim, FRG) and rabbit antihuman albumin (Behringwerke AG, Marburg/Lahn, FRG) were used. Serum was always diluted (1:400) with saline and both specimens (CSF and serum) were run in the same batch. IgM in CSF of CNS-SLE patients was measured by an ELISA method <sup>17</sup>, with slight modifications. In brief, plates were coated with  $150\text{ }\mu\text{l/well}$ , 1/500 diluted rabbit antihuman IgM in PBS-buffer, pH 7.4; DAKO Copenhagen.  $100\text{ }\mu\text{l}$  of CSF and (1:10) diluted CSF and human IgM standards (Behringwerke AG, Marburg/Lahn, FRG) in duplicate were used per well. Rabbit, peroxidase-conjugated, antihuman IgM (DAKO, P125) was used for the enzyme reaction. The sensitivity level was  $0.05\text{ }\mu\text{g/l}$ .

Q albumin is CSF albumin concentration  $\times 10^3$ /serum albumin concentration.

Q albumin was used as an indicator of blood CSF barrier (BCB) function. The CSF index was used as an indicator of intrathecal synthesis of immunoglobulins and complement components. CSF IgG index = (CSF IgG concentration x serum albumin concentration)/(serum IgG concentration x CSF albumin concentration). CSF indices for IgA, IgM, C3, C4, and factor B were calculated by the same equation. The following reference values have been established in our laboratory. Q albumin < 7.0. For normal CSF total protein concentration, reference values for IgG index are 0.36 - 0.56, for IgA index 0.13 - 0.37, and IgM index <0.06. For a CSF total protein concentration ranging between 450 - 700 mg/l these values are 0.36 - 0.62 for IgG index, 0.16 - 0.40 for IgA index, and <0.10 for IgM index.

The Mann-Whitney U-test was used to compare patients and controls. In addition, reference values for C3, C4, and factor B concentrations in serum and CSF, and for the respective indices were computed using the normal order statistics. It was not possible to construct 90%-confidence intervals because of the small number of controls. Only the inner limits of these intervals could be estimated. Values between the inner limits may be considered normal.

## RESULTS

In the 12 patients with diffuse CNS-SLE, C4 indices were significantly elevated when compared with those of controls (median, 2.3 versus 1.1,  $p = 0.01$ ) (Figure 1). C3 and factor B indices were not significantly different (median, 0.6 versus 0.5,  $p = 0.1$ ; and 0.8 versus 0.7,  $p = 0.2$ ) (Figure 1). Reference values were 0.4 -0.7 for C3 index, 0.6 -1.6 for C4 index, and 0.6 -0.8 for factor B index. Six patients had normal CSF C3 indices, 4 had normal CSF C4 indices, and 6 had normal CSF factor B indices.

In patients and controls the CSF concentrations of C3 (median, 2.8 versus 3.2 mg/l,  $p = 0.8$ ), C4 (median, 1.8 versus 1.4 mg/l,  $p = 0.1$ ), and factor B (median, 1.0 versus 0.9 mg/l,  $p = 0.5$ ) did not differ significantly. Reference values were 2.0 -4.5 mg/l for CSF C3, 1.0 -1.8 mg/l for CSF C4, and 0.5 -1.5 mg/l for CSF factor B concentration. Six patients had normal CSF C3 concentrations, 4 had normal CSF C4

concentrations, and 8 had normal CSF factor B concentrations.

The serum concentrations of C3 (median, 960 versus 1214 mg/l,  $p = 0.08$ ) and of factor B (median, 248 versus 252,  $p = 0.9$ ), were not significantly different between patients and controls. Serum C4 concentrations in the patient group (median 158 mg/l) were significantly lower than in controls (median 245 mg/l,  $p = 0.02$ ). Serum reference values as obtained in controls were 978 -1606 mg/l for C3, 158 -284 mg/l for C4, and 207 -364 mg/l for factor B. Six patients had normal serum C3 concentrations, 4 had normal serum C4 concentrations, and 9 had normal serum factor B concentrations.

In patients 4 and 8 (Table 1) an increased Q albumin (14.2 and 8.7) existed, indicating impairment of the BCB. In two patients (9 and 12) the IgG index was increased, and in one patient (9) the IgA index. Eight patients had an increased IgM index (Table 1).

Table 1: Neuropsychiatric diagnosis, CSF and serum C4 concentrations, C4 index, and IgM index of 12 patients with active diffuse CNS-SLE.

N°/age in years/sex	neuropsychiatric diagnosis	C4	C4	C4	IgM
		CSF (mg/l)	serum (mg/l)	index	index
1/37/M	OPS, CI	2.5**	120*	3.50**	0.09**
2/30/F	D, CI	2.0**	125*	3.30**	0.08**
3/29/F	GS	1.2	40*	5.37**	0.18**
4/49/M	GS	1.6	239	0.48	0.05
5/42/F	OPS	0.6*	160	0.87	0.05
6/26/F	CI, GS	0.9*	156*	1.88**	0.05
7/55/F	D, CI	1.7	500**	0.86	0.15**
8/61/M	GS	4.7**	476**	1.15	0.05
9/44/F	D	3.2**	190	2.43**	0.19**
10/45/F	D	1.2	148*	2.52**	0.15**
11/24/F	OPS	2.1**	170	4.11**	0.44**
12/17/F	GS	1.9**	95*	6.31**	0.07**

Legend: OPS = organic personality syndrome; CI = cognitive impairment; D = delirium; GS = generalized seizures; \* = value below lower limit; \*\* = value above upper limit.

For reference values see section Methods and Results.

No relation was seen between type of neuropsychiatric diagnosis and increase of indices, especially the C4 index.



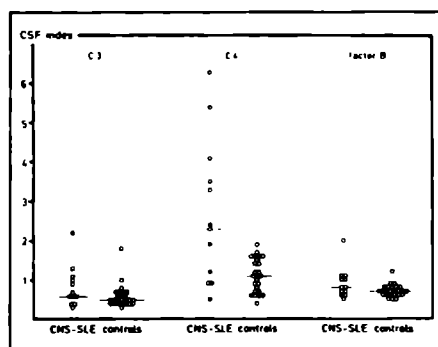


Figure 1: CSF indices for complement components C3, C4, and factor B in patients with active diffuse CNS-SLE ( $n = 12$ ) and in controls ( $n = 25$ ). Bars denote median values.

Comparing C3, C4, and factor B indices with immunoglobulin indices, the C4 indices in patients with an increased IgM index ( $n=8$ ) were significantly higher than in patients with a normal IgM index ( $n=4$ ) (median, 3.4 versus 1.0,  $p = 0.03$ ). Moreover, the C4 index was normal in 3 of the 4 patients with a normal IgM index, and in only 1 of the 8 patients with an increased IgM index (Table 1, Figure 1).

## DISCUSSION

In this prospective study, we demonstrated increased C4 indices in patients with active diffuse CNS-SLE, as compared to controls, and found significantly higher C4 indices in patients with an increased IgM index. There was no relation between the specific neuropsychiatric diagnoses and C4 index. C3 and factor B indices were normal.

Calculation of indices is a valid method for the detection of intrathecal immunoglobulin synthesis in autoimmune diseases of the CNS<sup>18, 19, 20</sup>, and complement indices are used to demonstrate intrathecal complement synthesis<sup>14, 21, 22</sup>. The ranges of complement index reference values we found are quite comparable to those found by others<sup>14, 21, 22</sup>. However, the blood - CSF exchange rates of individual proteins are different<sup>23</sup>. Following intravenous injection, it takes 20 hrs for albumin, and 3 to 6 days for IgG, to reach an equilibrium in the CSF<sup>24, 25</sup>. So, a fast decrease of the serum C4 concentration, e.g. due to massive consumption, may increase the C4

index value during the equilibrium period. In fact, we found significantly lower serum C4 concentrations in our patients, thereby confirming previous reports <sup>9, 12, 13</sup>. However, in SLE the greatest decrease in serum C4 occurs early in exacerbation <sup>26</sup>, and in our patients CSF specimens were obtained when neuropsychiatric manifestations had stabilized for at least 10 to 14 days. Therefore, we think that in our patients the increased C4 indices actually do reflect intrathecal C4 synthesis.

Monocytes/macrophages are the major extrahepatic source of complement components <sup>14</sup>. Recently, Passwell et al. reported an increased renal expression of the complement genes C2, C3, C4, and factor B, and an increased renal C3 and factor B synthesis in murine lupus nephritis <sup>27</sup>; brain tissue was not examined. Mallat and Levi-Strauss demonstrated C3 production in primary culture of rodent brain astrocytes, and synthesis of C3 and factor B was demonstrated using clonal cell lines belonging to the astrocyte lineage <sup>28, 29</sup>. In the light of these experimental data, the intrathecal C4 synthesis in our patients suggests activation of astrocytes or intracerebral monocytes/macrophages in active diffuse CNS-SLE.

C3 and factor B indices did not differ significantly from those in controls. This finding could be related to the stage of the disease process at the time of examination. On the other hand, C3 and factor B might be less involved in CNS-SLE than C4, since in active SLE there are usually marked depressions of serum C4 concentrations, whereas serum C3 and factor B concentrations are less substantially decreased, or even normal <sup>8, 10</sup>. As far as factor B is concerned, it should be stressed that the classical complement pathway, and not the alternative one, is predominantly involved in SLE <sup>1</sup>.

C4 indices were significantly higher in patients with an increased IgM index. As mentioned, IC occur in CSF of CNS-SLE patients <sup>7</sup>. Given an activation of the classical complement pathway in our patients, as the increased C4 indices suggest, IC containing C3 split products could have stimulated B cells <sup>30</sup>, leading to intrathecal IgM synthesis.

As far as we know, we are the first to demonstrate intrathecal C4 synthesis, related to intrathecal IgM production, in patients with diffuse CNS-SLE, suggesting that IgM antibodies and C4 might cooperate in diffuse CNS-SLE pathogenesis.

However, our data do not justify the use of the C4 index as an indicator of diffuse CNS-SLE disease activity in individual patients, nor do they inform about the potential value of the C4 index in distinguishing diffuse CNS-SLE from steroid-induced delirium or CNS infection. Given the advantages of Ig CSF indices over CSF concentrations in CNS-SLE patients <sup>6</sup>, serial studies are needed to examine the usefulness of the C4 index in diagnosing diffuse CNS-SLE and in monitoring diffuse CNS-SLE disease activity.

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## **CHAPTER 6**

### **SUMMARY, DISCUSSION AND DEVELOPMENTS**

## SUMMARY

Cerebrospinal fluid (CSF) analysis has received increasing interest for studying (abnormal) brain metabolism and for diagnosing diseases of the central nervous system (CNS). In the present study we investigated the relevance of CSF analysis for the diagnosis of neurological diseases.

Chapter 2 gives an overview of various components in CSF and outlines the significance of these components for the differential diagnosis of neurological diseases. Investigation of cells, albumin ratio, glucose, lactate, Ig indices and isoelectric focusing (IEF) are especially indicated for the (differential) diagnosis of (sub)acute infections of the CNS and for chronic inflammatory neurological diseases, such as multiple sclerosis (MS) and autoimmune diseases with cerebral involvement. CSF cell and blood pigment analyses are important for diagnosing and distinguishing between haemorrhagic and non-haemorrhagic CNS infarcts. The microscopic observation of malignant cells in CSF and the determination of tumour markers are indicated for the diagnosis of brain metastases and for monitoring the effect of oncological therapy.

A strategy for CSF investigation is proposed. The relevant CSF components are subdivided into diagnostic groups and each group comprises a number of coherent CSF components. In our opinion, this design will help the clinician to select the appropriate CSF tests for making a diagnosis.

Chapter 3 reports on metabolic investigations in CSF and blood. During prolonged fasting in reference children, we observed a complex of interrelated metabolic processes to regulate the blood glucose level and to generate alternative energy substrates. Our finding of a clear interrelationship in blood between glucose, ketones and the age of the subject during prolonged fasting, supports the concept of diminished glucose homeostasis and increased ketogenesis in younger children compared to older ones (Chapter 3 section 1). The levels of glucose and ketones in CSF after prolonged fasting showed a clear correlation with the age-related concomitant blood concentrations. It may be postulated that CSF:blood ratios give information about the transport process of substrates from blood to CSF/brain. The ratio for glucose and  $\beta$ -hydroxybutyrate was independent of the blood level or age. However,

the ratio for acetoacetate was significantly higher in the younger children than in the older ones, which indicates age-dependent acetoacetate transport. The CSF caloric values after prolonged fasting were independent of age. A lower glucose level in the CSF of the younger children was fully compensated for by a higher ketone level. This suggests that ketone bodies contribute to CSF caloric homeostasis and compensate for decreased glucose provision, especially in younger children (Chapter 3 section 2).

In section 3, we studied the levels of fuel-related components after prolonged fasting in the blood and CSF of children with encephalopathy of unknown origin and compared the results to those of the reference children. It was our aim to investigate whether there were any disturbances in the energy metabolism of these children, as it is known that the functional activity of the brain is related to the brain energy supply. After prolonged fasting, in the patients with mental retardation of unknown etiology, a significantly higher blood glucose level, compared to the reference patients, was found. Several factors could have been responsible for this phenomenon. It is possible that a reduction in the glucose metabolism/utilization of brain cells in these patients might be a major factor. In patients with complex partial epilepsy, the alterations were more pronounced. The lower CSF ketone values and CSF:blood ketone ratios in these patients suggest ketone transport disturbances from blood to brain or increased brain ketone consumption. The CSF abnormalities were not observed in a group of children with primary generalized epilepsy. We could not demonstrate any link between the alterations in ketone metabolism and the clinical conditions in patients with complex partial epilepsy.

Chapter 4 elaborates on the relevance of brain-specific proteins in CSF. The CSF levels of neuron-specific enolase (NSE), S-100 protein (S-100) and myelin basic protein (MBP) were found to be age-dependent. It was remarkable that the relative increases in NSE, S-100 and MBP with age were similar. Explanations for this phenomenon could be 1) an increase in neuron, glia and myelin loss with age, or 2) an unknown common underlying mechanism (Chapter 4 section 1). The behaviour of these proteins in the CSF of various young and adult neurological patients was also investigated. We did not observe any distinct pattern of brain-specific proteins in the different neurological disorders. The three CSF proteins seemed to behave depen-



dently in some of the disorders, especially in the acute cerebrovascular accident (CVA) patients, which would indicate more generalized brain involvement. No dependent behaviour was seen in the acute MS patients.

In CSF, signs of demyelination (MBP increase) were found much more frequently than neuronal damage (NSE increase), which may indicate that neurons are better protected against damage than myelin (Chapter 4 section 2).

Active MS patients had increased CSF MBP levels but these returned to normal after immunosuppressive treatment either with cyclophosphamide or with intravenous methylprednisolone, which indicates a reduction or stop in the demyelination process (Chapter 4 sections 3 and 4). The mean CSF MBP level was significantly higher in the relapsing-remitting (RR) MS patients and in the chronic progressive MS patients with relapses than in the chronic progressive MS patients without a RR disease course. A relationship was demonstrated between the clinical scoring (Kurtzke's Expanded Disability Status Scale (EDSS)) and the CSF MBP levels in RR MS patients. The number of gadolinium-enhancing lesions detected with MRI in active MS patients was significantly correlated with the CSF MBP level and a correlative triad was found between the decrease in gadolinium-enhancement, a decrease in CSF MBP and the clinical improvement after methylprednisolone treatment (Chapter 4 section 4). These observations stress the importance of CSF MBP in MS as a valid parameter for disease activity and may indicate that reduction of inflammation is accompanied by decrease in myelin breakdown and clinical recovery.

For the diagnosis of a particular neurological disease, brain-specific proteins are less relevant. However, they do show great promise for assessing the involved brain compartment, the extent of the lesion or the activity of the (neurological) disease process. An increased CSF level of (one of) these brain-specific proteins indicates brain damage, but a normal level does not exclude (minor) brain damage. Increased CSF levels of these proteins were found in patients who were suffering from an acute neurological disorder with brain damage, or from a chronic progressive neurological disorder. CSF determination of brain-specific proteins is not indicated in patients with chronic neurological diseases.

Chapter 5 describes the value of assessing the humoral and cellular immune response in CSF in some neurological diseases. One of the most important CSF tests is isoelectric focusing for the detection of oligoclonal gamma bands. These bands were observed almost exclusively in infectious, inflammatory and demyelinating diseases of the CNS. The CSF aberrations were caused by immunological reactions which caused the intrathecal production of microheterogeneous IgG compounds. In infectious neurological diseases, these abnormalities were observed much more frequently in the subacute and chronic forms than in the acute forms. In the CSF of MS patients, we frequently observed (92%) an oligoclonal gamma banding pattern, which indicated the presence of oligoclonal IgG-producing B-cells in the brain. The identity of these IgGs is unknown. Methylprednisolone treatment reduced the number of oligoclonal bands only slightly, indicating that eradication of IgG producing B-cells in brain has not really occurred (Chapter 5 section 1).

Increased IgG indices were frequently observed in MS patients and also in patients with systemic lupus erythematosus with diffuse CNS involvement (neuro-SLE) (Chapter 5 sections 1 and 3). Both of these diseases are probably autoimmune mediated and the increased IgG indices indicate persistent humoral immune activity in the brain of patients with these diseases. We did not find any correlation between CSF intrathecal IgG and the clinical scoring (EDSS) or demyelination (CSF MBP) in MS patients (Chapter 5 section 1). This absence might contradict the existence of a direct link between IgG-producing B-cell activity in the brain and the clinical activity. IgM was considered to be a more serious candidate for monitoring disease activity in MS. In RR MS patients, we observed a correlation between CSF intrathecal IgM and demyelination (CSF MBP) (Chapter 5 section 1). The finding of a correlation between the CSF IgM index and the complement component C4 index in our study on neuro-SLE patients might also support the assumption that IgM-producing B-cells in the brain are more directly involved in the (auto)immune-mediated inflammatory process than IgG-producing B-cells (Chapter 5 section 3). Methylprednisolone treatment significantly reduced intrathecal IgG and IgM in MS patients.

Isoelectric focusing of CSF proteins is strongly indicated in MS, (chronic) infections of the CNS and in autoimmune diseases of the CNS. IgG index gives

additional support for the monitoring/diagnosis of the above-mentioned diseases. IgM index is of more importance to determine the acuity and activity of the disease process.

Section 2 presents our observations on lymphocyte subsets in the CSF and blood of MS patients. In the active MS patients, the blood CD4+ (helper/inducer) T-cell percentage was significantly decreased, while the CSF CD4+ percentage tended to be higher in comparison with the controls. Furthermore, the number of mononuclear CSF cells and the percentage of CD20+ (B-cells) in CSF were significantly increased compared with the controls. The positive correlation between CSF mononuclear cell number and the CSF CD4+ T-cell percentage and the negative correlation between cell number and CSF CD8+ (suppressor / cytotoxic) T-cell percentage suggest that a selective increase of CD4+ T-lymphocytes accounts for the increased cellularity in the CSF and brain of active MS patients. No relationship was found between the various percentages of T-cell subsets in the CSF or blood of patients with different courses of the disease or with other clinical variables (EDSS, disease duration, progression rate). After treatment with intravenous methylprednisolone, the percentage of CD8+ T-cells was significantly decreased in blood and increased in CSF compared with the values before treatment. This phenomenon might suggest a selective traffic of CD8+ cells from the blood to the CSF and to the brain compartment, and this migration might affect the inflammatory process. It still has to be established whether determining lymphocyte subsets in patients with MS has any value for assessing an abnormal immune regulation within the CNS and monitoring treatment effects.

## DISCUSSION

Many neurological disorders are associated with changes in the composition of the CSF. It is beyond the scope of this thesis to give guidelines regarding indications for lumbar puncture (LP). CSF analysis at most hospital laboratories is restricted to a number of standard laboratory investigations (cells, protein content, glucose, electrophoresis, IgG index). Some university hospitals have specialized CSF laboratories

where additional "more specific" CSF investigations can be performed. These investigations become integrated into advanced CSF diagnostic tests once their clinical relevance has been proved.

### **Clinical relevance of CSF investigation**

#### **1. *Indicated for clinical diagnosis***

##### **● Acute infections of the CNS**

CSF investigation must be performed without delay in the case of a suspected meningitis syndrome. In many patients with fever of unknown origin, even in the absence of meningeal signs, CSF investigation can be of great value as a guide to both diagnosis and treatment. Furthermore, in the early stages of CNS infection (bacterial, viral, fungal or protozoal) it is not possible to make a differential diagnosis without CSF analysis. Such an analysis should include CSF leukocyte number, cell differentiation, glucose, lactate, total protein and brain specific protein measurements. For the detection of antigens, special tests are available (Elisa, Latex agglutination and recently the use of the polymerase chain reaction (PCR)). CSF investigation is also indicated in the case of suspected encephalitis. If the patient is found to have Herpes Simplex encephalitis, treatment must be started as soon as possible.

##### **● Chronic inflammatory diseases**

For the differential diagnosis of chronic inflammatory diseases, the following questions must be answered: Is the process restricted to the nervous system or does it form part of a systemic disease? Is it caused by an exogen agent or does autoimmunity play a prominent role? Investigation of oligoclonal bands, Ig indices and specific antibodies (index) is indicated besides the standard tests. In chronic infections such as neuroborreliosis, neurosyphilis, parasitic infections, etc., oligoclonal bands and specific antibodies are often observed, including antibodies to opportunistic infections in immune compromised patients. In autoimmune-mediated disorders, (non)specific immunological abnormalities are observed in CSF in diseases such as cerebral vasculitis, neuro-SLE, neuro-sarcoidosis and neuro-Behçet.

CSF investigation is very important for the diagnosis of MS. Isoelectric focusing is the most sensitive test in CSF. The detection of oligoclonal bands supports the

diagnosis of MS and the absence of these bands in MS is rare. An European consensus article will be published about recommended CSF investigations in MS (see Addendum).

- Brain metastases

CSF investigation is indicated for the diagnosis of metastases into the CNS. Besides the observation of malignant cells in CSF, the determination of tumour markers is a particularly sensitive test.

- Inflammatory polyneuropathies

CSF investigation is indicated if Guillain-Barré's syndrome or chronic inflammatory demyelinating disease are suspected.

- Neurometabolic diseases

CSF investigation is indicated in some neurometabolic diseases, such as mitochondrial encephalomyopathies, non-ketotic hyperglycinemia, hepatic encephalopathy and encephalopathy of cause ignota.

## *2. Important help for diagnosis*

In cases of suspected subarachnoid haemorrhage, CSF investigation is indicated if the CT scan is negative or if there is some delay before the patient is admitted to hospital (> 12 hours) after the ictus. CSF analysis can also be helpful in some patients with a CVA to exclude inflammation, in benign intracranial hypertension and normal pressure hydrocephalus and in patients with a radicular syndrome to exclude underlying infection.

## *3. To assess the activity of the disease process, the prognosis and treatment effects*

CSF investigation of brain-specific proteins can give important information about the severity of the brain damage, the prognosis and treatment effects in diseases such as infections, MS and CVA. Neurotransmitter metabolites can be analysed to evaluate the involvement of the brain compartment, the prognosis and to regulate and monitor medication in diseases such as extrapyramidal disorders.

#### *4. Tool for research*

In various neurological disorders, CSF investigation can serve as a tool for research. CSF investigation can be expected to yield important information in neuro-immunological, neurometabolic and demyelinating disorders and in dementia. Several new CSF investigations have already reached an advanced research stage. Various developments show great promise for assessing the immune-mediated origin of a disease, the activity of a disease process and the possible underlying pathophysiology and for making a diagnosis.

- Cytokines

Cytokines are produced by a variety of cells, such as monocytes/ macrophages, lymphocytes, vascular endothelial cells and also astrocytes and microglia cells of the brain. These products stimulate cell replication, cell differentiation and immunoglobulin production of B-cells. Determination in CSF can offer information about ongoing intrathecal immunological activity. In active MS patients, increased levels of TNF- $\alpha$ , IL-2 and soluble CD8 (suppressor T-cell activator) were observed in contrast to stable MS patients [1-4]. In human immunodeficiency virus type 1 (HIV-1) infected patients, TNF- $\alpha$  levels were found to be increased [5]. Intrathecal production of cytokine IL-6 has also been established in infectious neurological diseases, such as viral and bacterial infection and cytokine TNF- $\alpha$  in cerebral malignancies, such as CNS leukaemia [6-8]. Cytokine determination does not make any significant contribution to diagnosis, but it does look promising as a possible marker for the immune-mediated status and it may be useful to evaluate the activity of a disease and for therapeutic approaches.

- Anti-brain protein antibodies

Anti-MBP antibody levels in CSF have already proved to be useful as marker for demyelination. It appears that T-lymphocytes respond to MBP and are involved in the pathogenesis of inflammatory demyelination [9]. Anti-MBP antibodies have been detected in active MS patients [10,11] and in patients with AIDS dementia complex [12]. Antibodies against myelin-associated glycoprotein (anti-MAG) have been observed in some patients with polyneuropathy associated with IgM paraproteinemia [13]. Further investigations on the specificity of this immune reaction and its correla-

tion with disease progression in demyelinating diseases will be worthwhile.

- **Lymphocytes coexpressing more surface antigens**

There is increasing evidence that chronic inflammatory processes, including autoimmune diseases, are mediated by imbalance of immunoregulatory cells. Several studies have indicated that T-cells in the CSF immune compartment represent populations of sequestered, activated cells with different immunological characteristics than T-cells in the peripheral blood.

Dual colour flow cytometry can define lymphocytes which coexpress several surface antigens. In the CSF of MS patients, increased levels of fetal-type CD5+ B-cells and CD4-8-T-cells and decreased levels of CD4+ 2H4+ (suppressor inducer) T-cells have been demonstrated [14,15]. These findings in CSF indicate the existence of an active immune system with low numbers of suppressor inducer T-cells in active MS which is accompanied by B-cell hyperactivity. In a patient with AIDS dementia complex, the level of CD8+ HLA-DR+ T-cells in CSF was increased suggesting an ongoing T-cell response to HIV in CNS [16].

- **Neurotransmitters (metabolites)**

Abnormalities in CSF monamine metabolites concentration are often demonstrated in extrapyramidal disorders, Alzheimer's disease and other diseases with dementia.

Recently, reduced CSF levels of 3-methoxy-4-hydroxyphenylethyleneglycol, 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) have been demonstrated in the primary fibromyalgia fibrositis syndrome (PFS), which supports the proposed hypothesis of a metabolic defect in PFS [17]. Furthermore, a new inborn error of metabolism due to aromatic L-amino acid decarboxylase deficiency has been reported with decreased levels of CSF HVA and 5HIAA in association with increased levels of 3-methoxytyrosine [18]. Assessment of cholinergic markers (acetylcholinesterase and butyrylcholinesterase) in CSF has revealed significantly reduced values in patients with various forms of dementia. An anomalous molecular form of acetylcholinesterase in CSF was reported in 11 out of 13 patients with Alzheimer's disease, but not in any of 10 non-demented individuals of a comparable age [19]. Amino acids such as glutamate, aspartate, gamma-amino butyric acid (GABA) and glycine, also

serve as neurotransmitters. Reduced CSF glycine levels have been reported in Alzheimer's disease [20]. Low CSF GABA levels were found in startle disease (hyperekplexia) which may suggest a genetic defect [21]. Inherited disorders of CNS GABA metabolism have been reported with both increased and decreased CSF GABA concentration [22].

- CNS methylation capacities

The reduced availability of methyl groups within the CNS can cause dysmyelination of the brain [23]. S-adenosylmethionine (SAM) is the sole methyl donor in many important methylation reactions in the CNS and involves neurotransmitters, (poly)amines, membrane phospholipids and proteins. Folate and vitamin B12 are involved in the formation of SAM. Long-term deficiencies of these vitamins can cause reduced SAM levels in the brain and dysmyelination [24]. L-Methylmalonyl-CoA mutase and methionine synthetase require vitamin B12 as cofactor for enzyme activity. Studies have shown that the CSF levels of methylmalonic acid and homocysteine are markedly elevated in patients with vitamin B12 deficiency [25-27]. The normal levels for vitamin B12 in CSF are very low. Therefore, measurement of CSF homocysteine and/or methylmalonic acid may be a better marker for vitamin B12 deficiency. Recently a study has appeared from our department about a neurological patient with a severe folate deficiency in CSF and a normal serum folate [28]. Besides vitamin deficiencies, inborn errors of the methyl transfer pathway can also cause low brain SAM levels [23]. Measurement of SAM in CSF might provide information on the methylation capacity of the brain. Decreased SAM levels in CSF were observed in several depressed patients and in patients with Alzheimer's disease [29]. The antidepressant effect of SAM administration has now been confirmed in several studies [29,30]. Children with an inborn error of the methyl transfer pathway also show low CSF SAM levels. Treatment with SAM has led to substantial clinical improvement, apparent remyelination and an increase in the CSF SAM level. Furthermore, it was recently reported that patients with ataxia of various etiologies had significantly decreased levels of CSF thiamine and thiamine monophosphate [31]. Other studies have also suggested thiamine involvement in several diseases characterized by cerebellar damage [32].



Measurement of vitamins or related products, such as homocysteine, methylmalonic acid and SAM, in CSF may provide information about the methylation capacity of the brain and possibly about the underlying pathophysiology.

- **Glycosphingolipids**

Glycosphingolipids are important constituents of all mammalian cell membranes, but are particularly abundant in brain tissue. The composition of the glycosphingolipids is cell type-specific. Increased release of plasma membrane glycosphingolipids to the intercellular space and the CSF might occur during degenerative processes and cell death in the brain. The CSF ganglioside pattern reflects some brain diseases [33]. The assay of sulphatide in CSF may provide important information about the extent and progress of demyelinating processes, such as those encountered in vascular and senile dementia and in MS [34].

## **DEVELOPMENTS**

### *Harmonisation of protocols for CSF analysis and interpretation*

In 1991, the Commission of the European Community on Multiple Sclerosis invited leading scientists working at relevant CSF laboratories in Europe to make recommendations regarding CSF tests to assist in the diagnosis of MS, and to publish a consensus article on this topic. More than 20 European scientists worked out a proposal for relevant CSF tests and in 1993 they will publish a consensus protocol based on certain agreed common methodologies for the analysis of CSF components, as well as recommendations regarding the interpretation of the analysis results (see Addendum)

The same group of researchers is now engaged in extending this CSF approach to the harmonisation of protocols for the diagnosis of inflammatory and infectious brain diseases with the aid of advanced immunospecific techniques.

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# **ADDENDUM**

## **THE ROLE OF CEREBROSPINAL FLUID ANALYSIS IN THE DIAGNOSIS OF MULTIPLE SCLEROSIS: A CONSENSUS REPORT**

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**SUBMITTED FOR PUBLICATION**

## SUMMARY

The authors from twelve European countries discussed a consensus about CSF analysis, specifically focused on the relevance of various techniques for the diagnosis of multiple sclerosis. As a Committee of the European Concerted Action for MS (Charcot Foundation), we organised five workshops on CSF Standards in MS. We report here a sequence of recommended CSF investigations most relevant for patients who could have multiple sclerosis:

1. The most sensitive method for the detection of oligoclonal bands is isoelectric focusing (IEF). The best procedure is to use the same amounts of IgG in parallel CSF/serum samples and to reveal oligoclonal bands by IgG-specific antibody staining.
2. Any IEF test used for the diagnosis of multiple sclerosis must be checked at least once per year using "blind" standards for the five different CSF/serum patterns.
3. Quantitative measures of IgG for "increased CNS production" are less sensitive than isoelectric focusing.
4. The preferred method for detection of blood-CSF barrier dysfunction is the albumin quotient while CSF albumin or total protein is of secondary preference. These values must be interpreted with reference to the age of the patient.
5. Cells should be counted. The normal value is no more than 4 per microlitre.
6. The combined local synthesis of antibodies against measles, rubella and/or varicella zoster could represent a significant advance if this offers higher specificity (not sensitivity) for chronic rather than acute inflammation. Other tests which may prove to have useful correlations with clinical parameters include: IgM, IgA, free light chains or myelin basic protein.

## INTRODUCTION

The diagnosis of multiple sclerosis (MS) can be extended by use of modern techniques for the analysis of cerebrospinal fluid (CSF). This important fact has been previously acknowledged by another group of experts <sup>[1]</sup>. We have refined their notions of "oligoclonal bands or increased CNS production of IgG" and offer considered opinions on the relative importance of various analytical techniques. Specifically, we have contrasted qualitative (oligoclonal bands) with quantitative (various IgG/Alb ratios) methods for their relative sensitivity and/or specificity in the diagnosis of MS.

There are, unfortunately, several papers on MS which do not give sufficient technical details of the methods used for determination of oligoclonal bands and/or increased CNS production of IgG. These techniques must clearly be calibrated by each individual laboratory. To allow proper international comparisons, authors should specify what percentage of patients with clinically definite MS are positive using their techniques, i.e. sensitivity. They should also state what percentage of normal and/or other inflammatory diseases (acute vs. chronic) are positive, i.e. specificity.

### The Diagnosis of Multiple Sclerosis

This is ultimately a clinical process, but as noted above, CSF examination, among other paraclinical tests, is also important. Perhaps the greatest attraction of CSF examination (when compared with other paraclinical tests) is that it is the only test to primarily demonstrate the inflammatory origin of the abnormalities which may be localised by clinical, neurophysiological or MRI tests.

Amongst the various methods of CSF analysis, those which detect a humoral immune response within the central nervous system are the most important in MS. This is because local synthesis of IgG is reported in the vast majority (up to 95%) of MS patients.

Detection of CSF oligoclonal bands by isoelectric focusing is the most sensitive method to assess local synthesis when compared to all the quantitative analytical methods as well as other qualitative methods. It is not specific, however, as there may

be oligoclonal bands of IgG in any inflammatory neurological disease.

### Integrated Report

The results of several tests which give independent or complementary information, should be presented as patterns which specifically relate to various groups of neurological disorders in a clinically-orientated report. The clinician may benefit from graphical representation of such an integrated laboratory CSF protein report. It has to be emphasised that the time of lumbar puncture with respect to the course of the disease is important for the interpretation of CSF data.

## **SINGLE TOPICS OF CONSENSUS**

### Sample Handling and Cell Examination

A defined amount (about 10 mL in adults, less in children) of CSF should be collected in polypropylene, siliconised glass or glass tubes. Cell numbers should be counted as soon as possible (preferably within 30-60 minutes) after lumbar puncture (LP). If the LP is not performed at the same hospital as the CSF laboratory, the CSF should be transported to the CSF laboratory as soon as possible (preferably within 6 hours) for routine cytological examination. Differential cell loss occurs during CSF storage. The number of white cells in normal CSF should be no more than  $4/\mu\text{L}$  (see Reviews: Tourtellotte).

Cytological examination is considered complementary in the diagnosis of MS. About 50% of patients show a normal cell count and only 1% of MS patients have cell counts larger than  $35/\mu\text{L}$ . Such elevated cell counts make MS rather unlikely, and thus other diagnoses should be considered.

Different methods such as cytocentrifuge or sedimentation chamber can be used for cytological preparation <sup>[2]</sup>. The answer to the clinician should be descriptive including, if possible, considerations of any alternative diagnosis.

### Evaluation of the Blood-CSF Barrier

The blood-brain barrier and the blood-CSF barrier are different barriers. By

analysing the protein content of the lumbar CSF, it is possible to assess the integrity of the (functionally-defined) blood-CSF barrier (BCB), but not just the isolated blood-brain barrier (BBB). In healthy people and/or in patients without objective signs of neurological disorders, the passage of the plasma protein across the BCB depends upon their hydrodynamic radii under steady-state conditions <sup>[3]</sup>. In such reference populations the absolute CSF levels of plasma proteins depend on many factors like serum concentration, blood-CSF barrier function, CSF flow, molecular size, age of the patient and volume of CSF extracted.

Albumin, the major CSF protein, is synthesised only by hepatocytes and is not catabolised within the CNS. Dynamic studies using intravenously injected radio-labelled albumin <sup>[4]</sup> have demonstrated that serum albumin is the source of CSF albumin and strongly support the use of CSF/serum albumin quotients (QAlb = CSF albumin/serum albumin) to assess the BCB function. Another approach <sup>[5,6]</sup> is related to CSF albumin only: any increase of CSF albumin (above the mean) is indicative of a transudate.

Determination of total protein is less reliable than CSF albumin. The technique should in any case yield the same extinction coefficient for albumin as for IgG, due to the wide variability of IgG relative to albumin (e.g. between 3% and 30% total protein).

The QAlb is age-dependent <sup>[7,8]</sup>. The upper reference limit for the first 10 mL of lumbar fluid is  $5.0 \times 10^{-3}$  for patients under 15 years;  $6.5 \times 10^{-3}$  for patients under 40 years;  $8 \times 10^{-3}$  for patients under 60 years and  $8.9 \times 10^{-3}$  for older patients. Most patients with MS have QAlb values below the upper reference limit. Higher values suggest a different neurological disorder.

Transudated CSF immunoglobulins, as calculated by a CSF/serum quotient, are not linearly related to the QAlb in cases with BCB dysfunction. The use of non-linear formulae or graphs for the interpretation of IgG values is therefore recommended.

The albumin concentration in a standardised volume of CSF and in the serum collected simultaneously must be analysed with the same immunoassay within the same analytical series.



### Quantification of the Humoral Immune Response in Brain

The extraordinarily high sensitivity of isoelectric focusing for the detection of oligoclonal IgG fractions in the CSF is an important complementary qualitative method and is treated separately below.

It is a necessary requirement for any quantitative assay that each laboratory must establish their own reference range for particular tests.

The detection of a humoral immune response in the CNS requires an expression of results which will discriminate between blood-derived and brain-derived immunoglobulin fractions in CSF. Such quantitative expressions are based upon calculations of the CSF/serum quotients [4,6,9-11] and these expressions are also used for comparisons of intrathecal synthesis of the various immunoglobulin classes (IgG, IgA, IgM) as well as for calculation of specific antibody index values to try to characterise acute and chronic inflammatory diseases.

The quotient evaluation reduces the analytical variations for the concentrations of plasma proteins in CSF. The CSF/serum ratio of IgG also reduces the individual variation of serum IgG. By referring this CSF/serum IgG quotient to the CSF/serum albumin quotient it is possible to further reduce the variation of the IgG quotient related to individual differences in BCB function. There are many approaches by which both of these quotients are combined to obtain an expression which will discriminate between the locally-synthesised IgG fraction in brain and the fraction of CSF IgG which is derived from the blood by filtration.

The use of a non-linear relation between the IgG quotient and the albumin quotient is recommended, since a linear approach can lead to a loss of sensitivity when there is BCB dysfunction, especially for larger molecules such as IgA or IgM [11-14].

The graphic representation of the immunoglobulin quotients as a function of the albumin quotient is suggested as part of a report for the clinician, as this gives simultaneous information about any local humoral immune response and/or any BCB dysfunctions.

The quantitation of local IgG synthesis in CSF has gained further importance for differential diagnosis by its relation to the intrathecal synthesis of other

immunoglobulin classes, namely IgA and IgM. This is the consequence of the special neuroimmunological regulation which seems to have some independence from blood immunological responses. More or less typical patterns of immunological classes of reaction have been observed in various neurological diseases [6,15].

The detection of synthesis of specific antibodies in CSF (e.g. against measles virus) has gained some clinical relevance through improvement of the sensitivity of the evaluation methods, mainly by the introduction of extrapolation methods, which refer to interpolated values for the IgG (or IgM) quotient, including corrections for any BCB dysfunction [6,16-18]. Of special interest for the diagnosis of multiple sclerosis is the notion that polyspecific antibody synthesis can occur in the brain. Thus local synthesis (derived from interpolation) of measles, rubella and zoster antibody formation is found in the CSF of 80% of patients, in one series [18]. The relevance of these methods has been confirmed by the identification of oligoclonal patterns in isoelectric focusing for the single species (measles, rubella, zoster) by the affinity-mediated capillary blot technique [19]. In addition, the report that the antibody affinity is different in acute compared with chronic diseases, further supports the idea that the polyspecific immune response (polyclonal but unspecifically stimulated) may also become an important tool for diagnosis in multiple sclerosis [20].

For the correct interpretation of the humoral immune response in CSF, it is important to keep in mind that the local IgG, IgA or IgM synthesis including any specific antibody synthesis in the brain might have several origins. It could be due to a persistent antibody response of an old clinically irrelevant immunological process in contrast to an acute inflammatory process. Local IgG synthesis, detected either by increased IgG quotients or by isoelectric focusing, can still be seen many years following sufficiently treated cases of neurosyphilis or neuroborreliosis amongst other examples of an intrathecal immune response.

**Table 1** Frequencies of abnormal CSF parameters in clinically-definite MS

- Oligoclonal IgG in CSF (isoelectric focusing)	>95%
- Abnormal blood CSF barrier function (QA/b $> 7 \times 10^{-3}$ )	12%
- Increased IgG quotient (IgG index, IgG (local))	70-80%
- Increased cell count $> 4/\mu\text{L}$	50%

### Isoelectric Focusing of Oligoclonal IgG

Our strongest consensus is that isoelectric focusing (IEF) is the most sensitive test for multiple sclerosis using the same amounts of IgG in parallel CSF and serum specimens [5,13,19,21-31]. Bands are preferably visualised by IgG-specific antibody staining. In addition, useful information can be obtained on other proteins by using a general protein stain.

It should be emphasised that IEF is not a specific "marker" test for MS, but reaches its maximal value in differential diagnosis only when all other known causes of CNS inflammation have been excluded by performing other tests for known CNS antigenic stimuli.

The significance of individual bands in CSF can only be properly understood in the context of both a parallel serum specimen and with attention to the overall band pattern of all sample tracks on the IEF plate. A good practical indicator is to compare the serum patterns from different specimens - do they all have the "same" banding pattern? Common "same" bands are found uniformly at the same pI in all specimens of a given run and are due to artefacts produced by the ampholytes. The higher the number of these artefactual bands, the more difficult it is to recognise not only legitimate abnormal serum bands, but even CSF bands, which can be obscured by interference from the common bands.

Reports of CSF protein analysis to the clinicians must always clearly distinguish the facts from the interpretation and qualitative from quantitative results. Under "facts" it should be clear whether the band pattern in CSF is polyclonal (no bands) or monoclonal (paraprotein bands) or oligoclonal. There must be parallel investigation of serum with a clear comment on the relative band patterns in CSF and serum. Examples of the five types of patterns are shown in Figure 1 and Figure 2.

### **Banding patterns on isoelectric focusing**

These patterns in Figure 1 are simplified for purposes of demonstration only. Densitometric scanning is not required for interpretation, but can be used to enhance the band patterns.

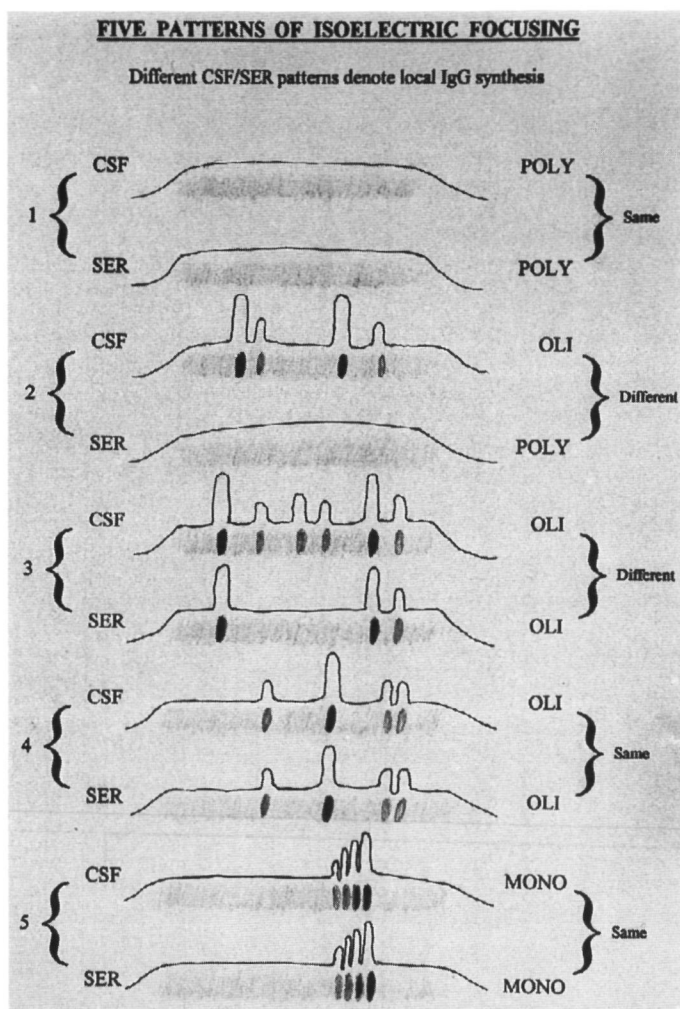


Figure 1. Idealised patterns for heuristic purposes are given.

Actual patterns are given in Figure 2 as examples of the 5 types. Original patterns are always more clearly visualised than any photographic reproductions.

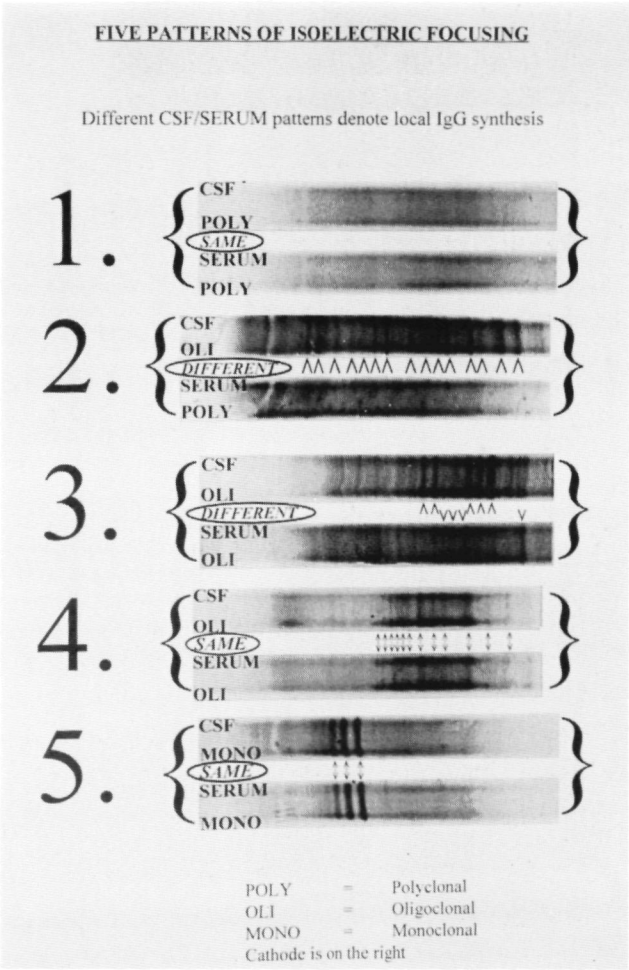


Figure 2. Typical patterns from individual cases are given.

**Evaluation - Remarks on the 5 types of band patterns**

Note that only patterns 2 and 3 represent local synthesis of IgG within the central nervous system.

1. Normal CSF.
2. CSF-restricted oligoclonal bands: local synthesis.
3. CSF-restricted oligoclonal bands with additional, identical bands in CSF and serum: local synthesis.

4. Identical bands in CSF and serum: not local synthesis.
5. Monoclonal bands in CSF and serum: not local synthesis.

### IgA

IgA analysis, either by quantitative or qualitative techniques, is of little value for the laboratory-supported diagnosis of MS.

Strong intrathecal IgA production may imply a different diagnosis. Most methods for quantitative analysis of IgA production have so far failed to take into account the relative proportion of monomeric and dimeric IgA in both CSF and serum, although dimeric IgA was shown to be preferentially produced in cases of intrathecal synthesis [32]. As a consequence, amounts of local IgA synthesis could be underestimated depending on the method used. The occurrence of oligoclonal IgA bands on isoelectric focusing in MS and/or other neurological diseases is not common [31,33].

### IgM

Determination of CSF IgM by quantitative and qualitative methods to demonstrate intrathecal production of IgM are optional tests for the diagnosis of MS. The recommended method for qualitative detection of oligoclonal IgM bands is electrophoresis or isoelectric focusing (agarose) of unconcentrated CSF and subsequent immunodetection [34]. Intrathecal production has been found, by quantitative and/or qualitative assays, in only 30-60% of MS patients and thus appears to be of less value than detection of oligoclonal IgG bands to assist the detection of a humoral immune response within the CNS. A degree of clinical relevance of IgM has been reported due to its decrease with duration of the disease process [34] and conversely, being more common with early exacerbations of the disease [21,36,37].

Further collaborative work is required to ascertain correlations between clinical variables and other CSF parameters including myelin basic protein [21,38].

### Free light chains

The detection of oligoclonal bands of free light chains in unconcentrated CSF can be performed by the same procedures as those used for demonstration of oligoclonal

IgG bands. In MS, presence of such oligoclonal free light chain bands is observed with about the same frequency as that for oligoclonal IgG bands, and this detection is a complementary, although optional, test to establish a laboratory-supported diagnosis [19]. Electrophoresis on polyacrylamide gel [39] or agarose [40] are alternative techniques to immunostain free light chains anodic to the gamma region.

The quantitative determination of free light chains is critically dependent on the specificity of the immunoassays used, as no reaction with bound light chain has to be observed with a 3 log IgG excess of bound versus free [41]. Absolute levels of free kappa and lambda chains are increased in about 80% and 60% of MS samples respectively [36]. The influence of both the serum levels and of BCB on the CSF levels are taken into account by the calculation of index values [41].

#### Quality assurance

Most of the standards for analytes in CSF diagnosis are defined as method-related values. It is necessary to use for internal quality assurance a reference material such as diluted serum or, much better, an accepted CSF control sample. For detection of imprecision a local CSF pool can be used as a daily control. External quality assessment (CSF survey) by an external agency [42] is also necessary. The International Standardising Organisations have co-operated to develop a harmonised proficiency testing protocol for CSF.

The special situation for CSF analysis is given by the advantage of calculating the CSF/serum quotient. If CSF and serum protein values are measured in the same run, the quotient formation eliminates many of the discrepancies due to method-related calibrations. The CSF/serum quotient thus approximates to a method-independent value.

The problem of a complicated quality control in cytology could be solved by sending sets of cytological slide preparations between different laboratories.

Proficiency testing in CSF analysis should also consider the control of the quality of data interpretation by including the five different focusing patterns [42] which have been widely recognised in our collaborative studies of "blind" CSF samples (data not shown).

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# **SAMENVATTING, DISCUSSIE EN ONTWIKKELINGEN**

## SAMENVATTING

De belangstelling voor het onderzoek van de cerebrospinale vloeistof (CSF) ter bestudering van de (abnormale) stofwisseling van de hersenen en voor de diagnostiek van ziekten van het centrale zenuwstelsel (CZS) is de laatste jaren duidelijk toegenomen. In deze studie hebben wij onderzoek gedaan naar het belang van CSF analyse voor de diagnostiek en prognostiek van neurologische ziekten.

Hoofdstuk 2 biedt een overzicht van de verschillende componenten in CSF en bediscussieert de betekenis van deze bestanddelen voor de differentiële diagnostiek van neurologische ziekten. Onderzoek van cellen, albumine quotiënt, glucose, lactaat, Ig indices en isoelectrische focusering (IEF) is vooral geïndiceerd bij de (differentiële) diagnostiek van (sub)acute infecties van het CZS en bij chronische neurologische ontstekingsziekten, zoals multiple sclerose (MS) en autoimmuun ziekten waarbij ook het brein betrokken is. Onderzoek van cellen en bloedpigmenten in CSF is belangrijk om onderscheid te maken tussen een herseninfarct met of zonder bloeding. Het aantreffen van kwaadaardige cellen in CSF en de aanwezigheid van verhoogde tumor merkstoffen in CSF wijzen naar een hersenmetastase en deze componenten kunnen belangrijk zijn voor het vervolgen van een medicamenteuze behandeling.

Een strategie voor CSF onderzoek wordt tevens voorgesteld. De verschillende CSF componenten zijn onderverdeeld in een aantal diagnostische groepen en iedere groep bestaat uit een aantal samenhangende CSF componenten. Naar onze mening zal deze strategie en het CSF aanvraagformulier de klinicus behulpzaam zijn bij het selecteren van de meest geschikte CSF onderzoeken.

Hoofdstuk 3 bediscussieert het onderzoek naar stofwisselingsproducten in CSF en bloed. Bij een groep referentie kinderen, die langdurig (40 uur) gehongerd hadden, konden wij een samenhangend complex van stofwisselingsprocessen vaststellen om de bloedglucosespiegel te reguleren en alternatieve energierijke substraten te genereren. Een relatie werd aangetoond in bloed tussen de concentraties van glucose en ketonen met de leeftijd na langdurig hongeren. Deze samenhang wijst erop dat jonge kinderen in tegenstelling tot oudere kinderen de bloedglucose concentratie slechter kunnen handhaven en sneller overschakelen op ketonen productie tijdens hongeren (hoofd-

stuk 3, sectie 1). De concentraties van glucose en ketonen in CSF na langdurig hongeren zijn duidelijk gecorreleerd met de leeftijdsafhankelijke bloedwaarden. De verhouding tussen de concentratie in CSF en bloed (CSF:bloed ratio) voor een bepaalde component kan informatie verschaffen over het transportproces van deze stof vanuit bloed naar CSF/hersenen. De CSF:bloed ratio voor glucose en  $\beta$ -hydroxyboterzuur was onafhankelijk van de bloedspiegel en van de leeftijd. De ratio voor acetylazijnzuur was significant hoger bij jonge kinderen vergeleken met oudere kinderen. Dit wijst naar een leeftijdsafhankelijkheid van acetylazijnzuur transport bij kinderen. De calorische waarde van de CSF na langdurig hongeren was gelijk voor alle leeftijden. De lage glucose concentratie in CSF bij jonge kinderen werd gecompenseerd door een hoge ketonen concentratie. Deze bevinding suggereert dat ketonen bijdragen aan de calorische homeostase van de CSF en voor de energievoorziening van de hersenen compensatie bieden voor het lage glucose niveau bij jonge kinderen tijdens hongeren (hoofdstuk 3, sectie 2).

In hoofdstuk 3, sectie 3 bestudeerden wij de concentraties van energierijke substraten in bloed en CSF na langdurig hongeren bij kinderen met encephalopathieën van onbekende origine en wij vergeleken de resultaten met die bij de referentie kinderen. Wij onderzochten of bij deze kinderen stoornissen in de energiestofwisseling voorkomen, aangezien bekend is dat de functionele activiteit van het brein mede afhankelijk is van de energievoorziening van de hersenen. Bij patiënten met mentale retardatie van onbekende oorsprong vonden wij na langdurig hongeren een significant hogere bloedglucose waarde in vergelijking met referentie kinderen. Verschillende factoren kunnen hiervoor verantwoordelijk zijn. Een verlaging in de stofwisseling/verbruik van glucose door hersencellen bij deze patiënten zou een belangrijke oorzaak kunnen zijn. Bij patiënten met complex partiële epilepsie waren de afwijkingen in bepaalde substraten nog meer uitgesproken. De lagere CSF ketonen waarden en de lage CSF/bloed ketonen ratio's suggereren stoornissen in het ketonen transport vanuit bloed naar de hersenen danwel toegenomen verbruik van ketonen in hersenen bij deze kinderen. Deze afwijkingen werden niet gevonden in een groep kinderen met primair gegeneraliseerde epilepsie. Wij konden geen verband ontdekken tussen de biochemische afwijkingen en de klinische afwijkingen in patiëntjes met complex

partiële epilepsie.

In hoofdstuk 4 wordt het belang van hersenspecifieke eiwitten in CSF nader uitgewerkt. De concentraties in CSF van neuron-specifieke enolase (NSE), S-100 eiwit (S-100) en myeline basisch eiwit (MBP) bleken leeftijdsafhankelijk te zijn. Het was opvallend dat de relatieve toename per jaar voor deze 3 eiwitten gelijk is. Verklaringen voor dit fenomeen zouden kunnen zijn 1) een met de leeftijd toenemend verlies van neuronen, gliacellen en myeline, 2) een nog onbekende gemeenschappelijke factor (hoofdstuk 4, sectie 1). Wij bestudeerden ook het voorkomen van deze eiwitten in de CSF bij jeugdige en volwassen neurologische patiënten. Wij konden geen bepaald patroon voor deze hersenspecifieke eiwitten vaststellen bij verschillende neurologische ziektebeelden. De drie betrokken eiwitten vertoonden wel een onderlinge afhankelijkheid bij bepaalde neurologische ziekten, met name bij patiënten met een cerebrovasculair accident (CVA), hetgeen wijst op een meer gegeneraliseerde aandoening van de hersenen. Deze afhankelijkheid werd niet geconstateerd in MS-patiënten met een actief ziektebeeld.

Een aanwijzing voor demyelinisatie (toename in MBP) werd in CSF veel frequenter gevonden bij neurologische patiënten dan een aanwijzing voor neuronen beschadiging (toename in NSE). Dit zou kunnen betekenen dat bij hersenschade de neuronen beter beschermd zijn dan myelinen (hoofdstuk 4, sectie 2).

MS-patiënten vertoonden in een actief stadium van de ziekte dikwijls een verhoogde MBP concentratie in CSF. Na een immuunsuppressieve behandeling met cyclofosfamide danwel intraveneus methylprednisolon (IVMP) normaliseerden de MBP waarden in CSF. Dit wijst op een reductie of beëindiging van het demyelinisatie proces (hoofdstuk 4, secties 3 en 4). De gemiddelde MBP concentratie in CSF was significant hoger in MS-patiënten met een exacerberende-remitterende (RR) vorm en in MS-patiënten met een chronisch progressief ziekteverloop gepaard gaande met exacerbaties en remissies (CP+RR) dan in chronisch progressieve MS-patiënten zonder een RR ziekteverloop. Wij konden een correlatie aantonen tussen de klinische score (volgens de Kurtzke's Disability Status Scale (EDSS)) en de MBP waarde in CSF in RR MS-patiënten. Magnetische resonantie afbeeldingen (MRI) met contrast (gadolinium-DTPA) van de hersenen van MS-patiënten tonen dikwijls nieuwe laesies.

Het aantal gadolinium-aankleuringen (laesies) in actieve MS-patiënten was significant gecorreleerd met het MBP gehalte in CSF en een gecorreleerd trias werd gevonden tussen afname in gadolinium-aankleuring, door middel van MRI, reductie in CSF MBP en klinische verbetering van de patiënt na IVMP behandeling (Hoofdstuk 4, sectie 4). Deze waarnemingen ondersteunen het belang van MBP in CSF als een valide parameter voor de activiteit van het ziekteproces en suggereren dat een vermindering van de ontsteking gepaard gaat met afname van myeline afbraak en klinische verbetering.

Hersenspecifieke eiwitten zijn minder relevant voor het stellen van de diagnose van een bepaalde neurologische ziekte. Echter, deze eiwitten kunnen belangrijke informatie verschaffen over het betrokken hersencompartiment, de uitgebreidheid van de laesie en/of de activiteit van het ziekteproces. Een verhoogde CSF spiegel van (één van) deze hersenspecifieke eiwitten wijst op hersenweefsel beschadiging. Een normaal gehalte sluit een (lichte) beschadiging niet uit. Verhoogde CSF spiegels van deze eiwitten worden aangetroffen bij patiënten, die een acute neurologische ziekte doormaken dan wel bij patiënten met een chronisch progressieve verloop van de ziekte. Hersenspecifieke eiwitten zijn niet geïndiceerd bij patiënten met een chronische neurologische ziekte.

Hoofdstuk 5 beschrijft de waarde van het vaststellen van een humorale of cellulaire immuunreactie in CSF bij bepaalde neurologische ziekten. Een van de belangrijkste testen is de isoelectrische focusering voor het aantonen van oligoclonale gamma banden. Deze gamma banden worden bijna uitsluitend aangetroffen bij infectieuze-, ontstekings- en demyeliniserende ziekten van het CZS. Deze afwijkingen worden veroorzaakt door immunologische reacties binnen het CZS die tot gevolg hebben een intrathecale productie van heterogene IgG componenten. Oligoclonale banden worden bij infectieziekten veel frequenter aangetroffen tijdens de subacute- of chronische fase van het ziekteproces vergeleken met de acute fase. Wij toonden bij MS-patiënten in de CSF heel frequent (92%) oligoclonale gamma banden aan, die derhalve wijzen op de aanwezigheid van oligoclonale IgG-producerende B-cellen in de hersenen. Het is niet bekend waartegen deze IgG's gericht zijn. IVMP behandeling reduceerde het aantal gamma banden slechts gering hetgeen betekent dat volledige



uitschakeling van deze B-cel activiteit niet optreedt (hoofdstuk 5, sectie 1).

Verhoogde IgG indices werden frequent aangetoond in CSF van MS-patiënten en patiënten met systemische lupus erythematosus (SLE) met neurologische complicaties (neuro-SLE) (hoofdstuk 5, secties 1 en 3). Waarschijnlijk zijn beide ziekten autoimmuun gemedieerd waarbij de verhoogde IgG indices wijzen op een persisterende humorale immuunactiviteit in de hersenen. Wij konden geen correlatie vaststellen tussen CSF intratheaal IgG en de klinische score (EDSS) of de mate van demyelinisatie (CSF MBP) in MS-patiënten (hoofdstuk 5, sectie 1). Dit pleit tegen een direct verband tussen IgG producerende B-cel activiteit in de hersenen en de klinische activiteit. IgM lijkt een betere parameter voor de ziekteactiviteit bij MS. In RR MS-patiënten werd een correlatie aangetoond tussen CSF intratheaal IgM en de mate van demyelinisatie (CSF MBP) (hoofdstuk 5, sectie 1). Ook werd een correlatie aangetoond tussen CSF IgM index en CSF complement component C4 index in neuro-SLE patiënten (hoofdstuk 5, sectie 3). Deze waarnemingen doen vermoeden dat IgM producerende B-cellen in de hersenen meer direct betrokken zijn bij het (auto)immuun-gemedieerde ontstekingsproces dan IgG-producerende B-cellen. IVMP behandeling reduceerde de intrathecale IgG en IgM productie significant.

Isoelectrische focusering van CSF eiwitten is sterk geïndiceerd bij een verdenking op MS, (chronische) infecties, ontstekingen en autoimmuunziekten van het CZS. De IgG index geeft een verdere ondersteuning voor bovenvermelde aandoeningen. De IgM index geeft meer informatie over de activiteit van het ziekteproces.

In hoofdstuk 5, sectie 2 worden de resultaten vermeld van een studie naar lymphocyten subpopulaties in CSF en bloed van MS-patiënten. In actieve MS-patiënten was het bloed CD4+ (helper/inducer) T-cel percentage significant verlaagd, terwijl het CSF CD4+ T-cel percentage hoger was in vergelijking met controle patiënten. Het aantal mononucleaire cellen in CSF en het percentage CD20+ (B-cellen) in CSF waren significant verhoogd in vergelijking met controle patiënten. Een positieve correlatie tussen het aantal CSF mononucleaire cellen en het percentage CSF CD4+ T-cellen en een negatieve correlatie tussen het aantal CSF mononucleaire cellen en het percentage CSF CD8+ (suppressor/cytotoxische) T-cellen werden aangetoond. Deze waarnemingen suggereren dat de verhoogde celreactie in CSF en in

hersenen in MS-patiënten veroorzaakt wordt door een selectieve toename van CD4+ lymphocyten. Er kon geen correlatie worden gevonden tussen T- of B-cel populaties in CSF en bloed en de EDSS of de mate van ziekteprogressie van MS-patiënten. Na de IVMP behandeling was er een significante daling van CD8+ T-cel percentages in bloed en een toename in CSF vergeleken met de waarden vóór de behandeling. Deze waarnemingen zouden kunnen wijzen naar een selectieve migratie van CD8+ cellen vanuit bloed naar CSF en hersenen, waardoor het ontstekingsproces beïnvloed wordt. Verder onderzoek zal noodzakelijk zijn om de waarde van lymphocyten populatie studies in MS te bepalen met betrekking tot een abnormale immuunregulatie binnen het CZS en voor het vervolgen van een therapeutische behandeling.

## DISCUSSIE

Veel neurologische aandoeningen gaan gepaard met veranderingen in de samenstelling van de CSF. Het valt niet binnen de doelstelling van dit proefschrift richtlijnen te geven omtrent de indicaties voor een lumbaal punctie. Onderzoek van de CSF blijft in de meeste ziekenhuislaboratoria beperkt tot het uitvoeren van een aantal standaard laboratorium bepalingen (cellen, eiwit gehalte, glucose, eiwitelectroforese, serologie en bacteriologie). Sommige universitaire ziekenhuizen beschikken over een gespecialiseerd CSF laboratorium, waar additionele "meer specifieke" CSF onderzoeken uitgevoerd kunnen worden. Deze analyses worden geïntegreerd in geavanceerde diagnostische CSF testen in het geval de klinische relevantie bewezen is.

### Klinische relevantie voor CSF onderzoek

#### 1. *Geïndiceerd voor de klinische diagnose*

##### ● Acute infecties van het CZS

CSF onderzoek moet direct worden uitgevoerd in geval van verdenking op een meningitis syndroom. Bij veel patiënten met koorts van onbekende oorsprong kan, zelfs in afwezigheid van meningeale tekens, CSF onderzoek van grote waarde zijn voor diagnose en behandeling. Bovendien is het niet mogelijk om in de vroege fase van een infectie van het CZS (bacterie, virus, schimmel of protozoa) een differentiële

diagnose te stellen zonder CSF onderzoek. Dit CSF onderzoek zou de volgende analyses moeten bevatten: aantal leukocyten, celdifferentiatie, glucose, lactaat, totaal eiwit en hersenspecifieke eiwitten. Voor het aantonen van het verantwoordelijke antigeen zijn speciale testen beschikbaar o.a. Elisa, Latex agglutinatie en, zeer recent, de polymerase keten reactie (PCR). CSF onderzoek is ook geïndiceerd bij verdenking op een encephalitis. In geval van Herpes Simplex encephalitis moet behandeling zo snel mogelijk gestart worden.

- Chronische ontstekingsziekten

Bij de differentiële diagnostiek van chronische ontstekingsziekten moeten de volgende vragen beantwoord worden: Is het proces beperkt tot het CZS of maakt het deel uit van een systemische aandoening? Is een exogene substantie de oorzaak of speelt autoimmunititeit een overwegende rol? Onderzoek in CSF naar oligoclonale gamma banden, Ig indices en specifieke antilichamen (index) is geïndiceerd naast de standaard CSF testen. In chronische infectieziekten zoals: neuroborreliose, neurosyphilis en parasitaire infecties e.a. worden regelmatig in CSF oligoclonale gamma banden en specifieke antilichamen aangetroffen, inclusief antilichamen tegen opportunistische infecties in immuun-gecompromitteerde patiënten. In autoimmuun-gemedieerde aandoeningen kunnen in de CSF (niet)specifieke immunologische afwijkingen worden aangetroffen o.a. in cerebrale vasculitis, neuro-SLE, neurosarcoïdose en neuro-behçet.

CSF onderzoek is heel belangrijk voor de diagnose van MS. Isoelectrische focusering is de meest gevoelige test in CSF. Het aantonen van oligoclonale gamma banden ondersteunt de diagnose MS. Afwezigheid van deze banden wordt zelden gezien bij MS. Een Europees consensus artikel zal gepubliceerd worden over aanbevolen analyses in CSF bij MS (lees Addendum).

- Hersen metastasen

CSF onderzoek is geïndiceerd bij een verdenking op metastasen naar het CZS. Naast het onderzoek naar maligne cellen in CSF is vooral de bepaling van tumormerkstoffen een gevoelige test.

- Inflammatoire polyneuropathieën

CSF onderzoek is geïndiceerd bij verdenking op Guillain-Barré's syndroom of

chronische inflammatoire demyeliniserende ziekte.

- Neurometabole ziekten

CSF onderzoek is geïndiceerd bij bepaalde neurometabole ziekten zoals mitochondrieële encephalomyopathiën, niet-ketotische hyperglycinemie, hepatische encephalopathie en encephalopathie e causa ignota.

## 2. Belangrijke hulp bij de diagnose

Bij verdenking op een subarachnoidale bloeding is CSF onderzoek geïndiceerd als de CT-scan negatief is of als vertraging ontstaat bij de ziekenhuisopname (> 12 uur) na het incident. Bij sommige patiënten met een CVA kan CSF onderzoek informatief zijn met name ter uitsluiting van een ontsteking. Ook bij benige intracranieële hypertensie en bij normale druk hydrocephalus en bij patiënten met een radiculair syndroom (ter uitsluiting van een onderliggende infectie) kan CSF onderzoek nuttig zijn.

## 3. Ter beoordeling van de activiteit van het ziekteproces, de prognose en de behandelingseffecten

CSF onderzoek van hersenspecifieke eiwitten kan belangrijke informatie leveren over de ernst van de hersenaandoening, de prognose en de effecten van medicatie bij ziekten zoals infecties, MS en CVA. Neurotransmitter metabolieten in CSF kunnen bovenvermelde informatie verschaffen bij extrapyramidale stoornissen.

## 4. Middel voor research

CSF onderzoek kan bij verschillende neurologische aandoeningen dienen als middel voor research. Met name bij neuroimmunologische-, neurometabole- en demyeliniserende ziekten en bij dementie mag verwacht worden dat CSF onderzoek belangrijke informatie oplevert. Verschillende nieuwe ontwikkelingen in het CSF onderzoek zijn reeds veelbelovend v.w.b. het vaststellen van een immuun-gemedieerde oorsprong van een ziekte, de activiteit van het ziekteproces, de onderliggende pathofysiologie en voor het stellen van een diagnose.

- Cytokinen

Cytokinen worden geproduceerd door verschillende soorten cellen zoals monocysten/macrofagen, lymfocyten, vasculaire endotheelcellen en ook astrocyten en microgliacellen in de hersenen. Cytokinen stimuleren celdeling, celdifferentiatie en immuunglobulinen productie door B-cellen. Deze producten in CSF kunnen informatie bieden over voortgaande intrathecale immunologische activiteit. In actieve MS-patiënten werden verhoogde spiegels in CSF van TNF- $\alpha$ , IL-2 en oplosbaar CD8+ aangetoond in tegenstelling tot stabiele MS-patiënten [1-4]. Bij door humaan immuun deficiëntie virus type I (HIV-I) geïnfecteerde patiënten werden verhoogde TNF- $\alpha$  spiegels gemeten [5]. Intrathecale productie van cytokine IL-6 werd vastgesteld bij neurologische infectieziekten van virale en bacteriële oorsprong en TNF- $\alpha$  bij cerebrale maligniteiten zoals CZS leukemie [6-8]. Cytokinen metingen leveren geen bijdrage aan de diagnostiek maar zij zijn van belang als parameter voor de immuun-gemedieerde status van een ziekte, voor evaluatie van ziekteactiviteit en voor medicatie-effecten.

- **Anti-herseneiwit antilichamen**

Anti-MBP antilichaam spiegels in CSF hebben reeds hun nut bewezen als parameter voor demyelinisatie. T-lymfocyten bleken te reageren op MBP presentatie en waren betrokken bij de pathogenese van inflammatoire demyelinisatie [9]. Anti-MBP antilichamen werden aangetoond in CSF bij actieve MS-patiënten [10,11] en bij patiënten met AIDS dementie complex [12]. Antilichamen tegen myeline-geassocieerd glycoproteïne (anti-MAG) werden aangetroffen in sommige patiënten met polyneuropathie in associatie met IgM paraproteïnemie [13]. Nader onderzoek naar de specificiteit van deze immuunreactie en de relatie met de ziekte progressie kan waardevol zijn bij demyeliniserende ziekten.

- **Lymfocyten met co-expressie van meerdere oppervlakte antigenen**

Het wordt steeds meer duidelijk dat chronische inflammatoire ziekten inclusief autoimmuun ziekten gemedieerd worden door een verstoring in de regulatie van immuuncompetente cellen. Verscheidene studies hebben aangetoond dat T-cellen in het CSF compartiment een populatie van afgezonderde, geactiveerde cellen vertegenwoordigen met andere immunologische eigenschappen dan T-cellen in het perifere bloed.

Een dubbele kleur "flowcytometrie" techniek kan lymphocyten definiëren met co-expressie van meerdere oppervlakte antigenen. Verhoogde spiegels van foetaal-type CD5+ B-cellen en CD4-8-T-cellen en verlaagde spiegels van CD4+2H4+(suppressie inductie) T-cellen zijn aangetoond in de CSF van MS-patiënten [14,15]. Deze resultaten wijzen op het bestaan in actieve MS-patiënten van een actief immuun systeem met een laag aantal suppressie inductie T-cellen gepaard gaande met B-cel hyperactiviteit. Bij een patiënt met AIDS dementie complex was het percentage van CD8+ HLA-DR+ T-cellen in CSF verhoogd hetgeen suggestief is voor een voortgaande T-cel respons tegen HIV antigeen in het CZS [16].

- Neurotransmitters (metabolieten)

Abnormale concentraties van monamine metabolieten in CSF zijn dikwijls aangetoond bij patiënten met extrapyramidale aandoeningen, patiënten met de ziekte van Alzheimer en andere vormen van dementie.

Onlangs werden verlaagde spiegels van 3-methoxy-4-hydroxyphenylethyleenglycol, 5-hydroxyindolazijnzuur (5HIAA) en homovanilline zuur (HVA) aangetoond in CSF in het primaire fibromyalgie fibrositis syndroom (PFS), hetgeen steun verleent aan de hypothese van een metabool defect bij PFS [17]. Ook werd gerapporteerd over een nieuwe aangeboren stofwisselingsziekte ten gevolge van een aromatische L-aminozuur decarboxylase deficiëntie met verlaagde CSF-spiegels voor HVA en 5HIAA in associatie met een verhoogde 3-methoxytyrosine spiegel [18]. Bij patiënten met verschillende vormen van dementie werden significant verlaagde CSF waarden van cholinerge merkstoffen (acetylcholinesterase en butyrylcholinesterase) gevonden. Bij 11 van de 13 patiënten met de ziekte van Alzheimer werd een abnormale moleculaire structuur van het cholinesterase aangetoond terwijl bij 10 niet-demente personen van een vergelijkbare leeftijd dit fenomeen ontbrak [19]. Amino-zuren zoals glutamaat, aspartaat, gamma-amino boterzuur (GABA) en glycine kunnen ook werkzaam zijn als neurotransmittor. Bij de ziekte van Alzheimer werden verlaagde glycine spiegels in CSF gevonden. Lage CSF GABA-spiegels werden aangetoond in patiënten met hyperekplexia, hetgeen zou wijzen op een genetisch defect [21]. Aangeboren stoornissen in de GABA stofwisseling van de hersenen zijn gerapporteerd met zowel verhoogde als verlaagde GABA concentraties in CSF [22].

- Methyleringscapaciteit van de hersenen

Een verlaagde beschikbaarheid van methylgroepen in het CZS kan de oorzaak zijn van dysmyelinisatie van de hersenen [23]. S-adenosylmethionine (SAM) is de exclusieve methyl donor bij veel belangrijke methyleringsreacties in het CZS en is als zodanig betrokken bij de synthese van neurotransmitters, (poly)aminen, membraan fosfolipiden en proteïnen. Foliumzuur en vitamine B12 zijn nodig voor de synthese van SAM. Langdurige deficiëntie van deze vitaminen kan een verlaagde SAM-spiegel in de hersenen veroorzaken en dysmyelinisatie [24]. L-Methyl-malonyl-CoA mutase en methionine synthetase hebben vitamine B12 nodig als co-factor voor enzymactiviteit. Studies hebben aangetoond dat de CSF-spiegels van methylmalonzuur en homocysteïne duidelijk verhoogd zijn bij patiënten met een vitamine B12 deficiëntie [25-27]. De concentratie van vitamine B12 in CSF is zeer laag. De bepaling van homocysteïne en/of methylmalonzuur in CSF is hoogstwaarschijnlijk een betere methode voor het vaststellen van een vitamine B12 deficiëntie in CSF. Onlangs is een publicatie uitgewerkt van onze afdeling over een neurologische patiënt met een ernstige foliumzuur deficiëntie in CSF en een normaal serum foliumzuur [28]. Behalve vitamine deficiënties kunnen ook aangeboren defecten in de methyl overdracht stappen een lage SAM-spiegel in de hersenen veroorzaken [23]. Meting van SAM in CSF kan dus informatie verschaffen over de methyleringscapaciteit van de hersenen. Bij patiënten met depressies en bij patiënten met de ziekte van Alzheimer werden verlaagde spiegels van SAM in CSF aangetoond [29]. Het antidepressieve effect na toediening van SAM is nu in meerdere studies bevestigd [29,30]. Lage SAM-spiegels zijn ook aangetoond in CSF bij kinderen met een aangeboren defect in methyl overdracht reacties. SAM-toediening had een substantiële klinische verbetering, duidelijke remyelinisatie en een toename in de CSF SAM concentratie tot gevolg. Een recente studie toonde aan dat patiënten met ataxie van verschillende aetiologie significant verlaagde spiegels van thiamine en thiamine monofosfaat hadden in de CSF [31]. Ook een andere studie toonde aan dat thiamine betrokken is bij ziekten met cerebellaire stoornissen [32].

Onderzoek in CSF naar vitaminen of aanverwante producten zoals homocysteïne, methylmalonzuur en SAM kunnen informatie verschaffen over de

methyleringscapaciteit van de hersenen en over de mogelijke pathofysiologie van het ziekteproces.

- Glycosfingolipiden

Glycosfingolipiden zijn belangrijke bestanddelen van celmembranen. Deze lipiden zijn vooral aanwezig in hersenweefsel. De samenstelling van de glycosfingolipiden is specifiek voor een bepaald celtype. Bij degeneratie en afsterven van cellen in de hersenen kunnen verhoogde concentraties van membraan glycosfingolipiden aangetroffen worden in de intercellulaire ruimte en in de CSF. Sommige hersenziekten gaan gepaard met een bepaald gangliosiden patroon in de CSF [33]. Onderzoek naar sulfatiden in de CSF kan belangrijke informatie verschaffen over de uitgebreidheid en de voortgang van demyeliniserende processen o.a. in vasculaire dementie en in MS [34].

## ONTWIKKELINGEN

### *Harmonisatie van protocollen voor CSF analyse en interpretatie*

De Commissie voor MS-onderzoek van de Europese Gemeenschap heeft in 1991 een aantal Europese deskundigen, werkzaam in gespecialiseerde liquorlaboratoria, uitgenodigd om aanbevelingen op te stellen over relevant liquoronderzoek ter ondersteuning van de klinische diagnose MS en om een consensus artikel over dit onderwerp te publiceren. Meer dan 20 Europese wetenschappers hebben een voorstel uitgewerkt voor relevante CSF testen en in 1993 zal een consensus protocol gepubliceerd worden over een aantal overeengekomen methodieken voor de analyse van CSF componenten en over aanbevelingen betreffende de interpretatie van de analyse resultaten (lees Addendum).

Dezelfde groep wetenschappers zal haar werkzaamheden voor het CSF onderzoek uitbreiden met de harmonisatie van protocollen voor de diagnostiek van ontstekings- en infectieziekten van de hersenen met behulp van geavanceerde immunospecifieke technieken.





## DANKWOORD

Op deze plaats wil ik iedereen bedanken die op enigerwijze heeft bijgedragen aan de totstandkoming van dit proefschrift.

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Mevr. J. Abma-Hill wil ik bedanken voor correcties en verbeteringen van de Engelse taal in het manuscript.

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## CURRICULUM VITAE

De auteur van dit proefschrift werd op 4 januari 1939 geboren te Roermond. In 1958 behaalde hij het diploma Gymnasium-B aan het Bisschoppelijk College te Roermond. In 1958 ging hij Biologie (hoofdrichting Biochemie) studeren aan de Katholieke Universiteit te Nijmegen alwaar hij in januari 1966 het doctoraal examen behaalde. Aansluitend werd de militaire diensplicht vervuld in het Marine Hospitaal te Overveen in de rang van reserve-luitenant ter zee, tweede categorie. Aldaar startte hij met de opleiding tot erkend klinisch chemicus (opleider: Dr. Ebeling). In oktober 1967 werd hij aangesteld als stafid van het Instituut voor Neurologie van de Universiteit te Nijmegen op het klinisch chemisch laboratorium (hoofd laboratorium en opleider: Drs. J.C.N. Kok), waar hij zijn opleiding vervolgde. In augustus 1970 werd hij als erkend klinisch chemicus ingeschreven in het Register. Vanaf medio 1988 is hij waarnemend hoofd van het afdelingsgebonden laboratorium voor kindergeneeskunde & neurologie (hoofd: Prof.Dr.Ir. J.M.F. Trijbels) van het Academisch Ziekenhuis te Nijmegen.







# **STELLINGEN**

behorende bij het proefschrift

## **CEREBROSPINAL FLUID ANALYSIS metabolic, brain damage and immunologic aspects**

in het openbaar te verdedigen  
op 8 december 1993  
des namiddags te 3.30 uur precies

door

**K.J.B. Lamers**



## I

Bij de interpretatie van analyses van hersenspecifieke eiwitten in liquor dient rekening gehouden te worden met de leeftijdsafhankelijkheid van de referentiewaarden van deze eiwitten. (Dit proefschrift).

## II

Ketolichamen leveren bij kinderen tijdens hongeren een belangrijke bijdrage aan de calorische homeostase van de liquor en aan de energievoorziening van de hersenen. (Dit proefschrift).

## III

In het algemeen is voor een correcte interpretatie van een liquoruitslag simultaan bloedonderzoek noodzakelijk. (Dit proefschrift).

## IV

Isoelectrische focusering is de meest gevoelige methode voor het aantonen van oligoclonale gammabanden in de liquor. Deze methode dient dan ook toegepast te worden door de betrokken laboratoria. (Dit proefschrift).

## V

Geavanceerd liquoronderzoek dient in verband met de relatief hoge kosten en de vereiste kwaliteit uitgevoerd te worden door een beperkt aantal gespecialiseerde liquorlaboratoria. (Dit proefschrift).

## VI

Voor de biochemische diagnostiek van mitochondriële (encephalo-)myopathiën is de lactaatbepaling in liquor relevanter dan in bloed of urine. (In JMF Trijbels, Review, Eur J Pediatr 1993;152:178-184).

## VII

Milde hyperhomocysteinemie wordt in het algemeen niet veroorzaakt door heterozygotie van cystathionine synthase deficiëntie. (Boers et al, N Engl J Med 1993; 85, 313:709-715).

## VIII

Analyse van lipiden in urine sedimenten kan bij verdenking op een lysosomale stapelingsziekte een belangrijke aanvulling vormen op de enzymdiagnostiek en kan, in bepaalde gevallen, een alternatief zijn voor het nemen van een biopt. (J. de Jong et al, Abstracts of the 31<sup>st</sup> SSIEM, 1992 nr: P107).

## IX

Afdelingsgebonden laboratoria waarborgen specifieke deskundigheid op diagnostisch- en onderzoeksgebied van het betreffende moederspecialisme. Deze laboratoria dienen derhalve in academische ziekenhuizen gehandhaafd te blijven.





